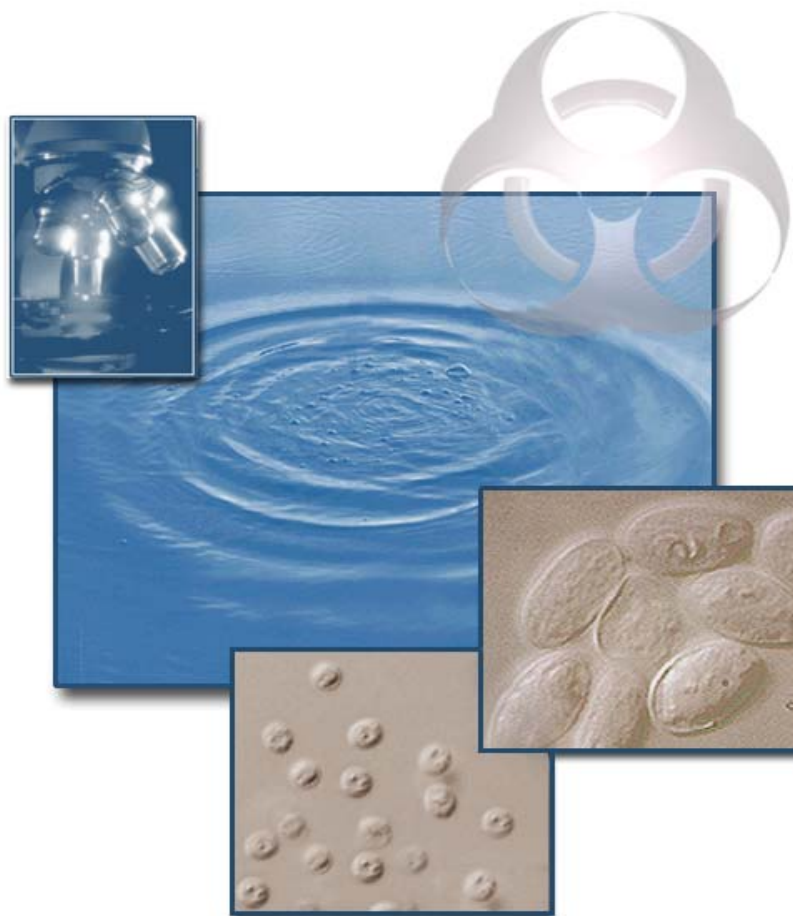




Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA

June 2003

Draft for Comment



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Cryptosporidium cover photo courtesy of the U.S. Centers for Disease Control

Giardia cover photo courtesy of CH Diagnostic & Consulting Service, Inc.

Disclaimer

This method has been reviewed by the U.S. EPA Office of Water and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Introduction

To support future regulation of protozoa in drinking water, the Safe Drinking Water Act Amendments of 1996 require the U.S. Environmental Protection Agency (EPA) to evaluate the risk to public health posed by drinking water contaminants, including waterborne parasites, such as *Cryptosporidium* and *Giardia*. To implement these requirements, EPA must assess *Cryptosporidium* and *Giardia* occurrence in raw surface waters used as source waters for drinking water treatment plants. EPA Method 1623 was developed to support this assessment.

Method Development and Validation

EPA initiated an effort in 1996 to identify new and innovative technologies for protozoan monitoring and analysis. After evaluating potential alternatives to the then-current method through literature searches, discussions with research and commercial laboratories, and meetings with experts in the field, the Engineering and Analysis Division within the Office of Science and Technology within EPA's Office of Water developed draft Method 1622 for *Cryptosporidium* detection in December 1996. This *Cryptosporidium*-only method was validated through an interlaboratory study in August 1998, and was revised as a final, valid method for detecting *Cryptosporidium* in water in January 1999.

Although development of an acceptable immunomagnetic separation system for *Giardia* lagged behind development of an acceptable system for *Cryptosporidium*, an acceptable system was identified in October 1998, and EPA validated a method for simultaneous detection of *Cryptosporidium* and *Giardia* in February 1999 and developed quality control (QC) acceptance criteria for the method based on this validation study. To avoid confusion with Method 1622, which already had been validated and was in use both domestically and internationally as a stand-alone *Cryptosporidium*-only detection method, EPA designated the new combined procedure EPA Method 1623.

The interlaboratory validated versions of Method 1622 (January 1999; EPA-821-R-99-001) and Method 1623 (April 1999; EPA-821-R-99-006) were used to analyze approximately 3,000 field and QC samples during the Information Collection Rule Supplemental Surveys (ICRSS) between March 1999 and February 2000. Method 1622 was used to analyze samples from March 1999 to mid-July 1999; Method 1623 was used from mid-July 1999 to February 2000. ~~The April 2001 revision of both methods include updated QC acceptance criteria based on analysis of the QC samples analyzed during the ICRSS.~~

Changes in the April 2001 Versions of the Methods

Both methods were revised in April 2001, after completion of the ICRSS and multiple meetings with researchers and experienced laboratory staff to discuss potential method updates. Changes incorporated in the April 2001 revisions of the methods (EPA-821-R-01-025 and EPA-821-R-01-026) included the following:

- Nationwide approval of modified versions of the methods using the following components:
 - (a) Whatman Nuclepore CrypTest™ filter
 - (b) IDEXX Filta-Max™ filter
 - (c) Waterborne Aqua-Glo™ G/C Direct FL antibody stain
 - (d) Waterborne Crypt-a-Glo™ and Giardi-a-Glo™ antibody stains
- Clarified sample acceptance criteria
- Modified capsule filter elution procedure
- Modified concentrate aspiration procedure

- Modified IMS acid dissociation procedure
- Updated QC acceptance criteria for IPR and OPR tests
- Addition of a troubleshooting section for QC failures
- Modified holding times
- Inclusion of flow cytometry–sorted spiking suspensions

Changes in the June 2003 Versions of the Methods

Both methods were revised again in June 2003 to support proposal of EPA's Long Term 2 Enhanced Surface Water Treatment Rule. Changes incorporated into the December 2002 versions include:

- Nationwide approval of a modified version of the methods using the Pall Gelman Envirochek™ HV filter
- Removal of Whatman Nuclepore CryptTest™ filter from the methods as a result of discontinuation of the product by the manufacturer
- Nationwide approval of the use of BTF EasySeed™ irradiated oocysts and cysts for use in routine quality control (QC) samples
- Minor clarifications and corrections
- Rejection criteria for sample condition upon receipt
- Guidance on measuring sample temperatures
- Clarification of QC sample requirements and use of QC sample results
- Guidance on minimizing carry-over debris onto microscope slides after IMS

Performance-Based Method Concept and Modifications Approved for Nationwide Use

EPA Method 1623 is a performance-based method applicable to the determination of *Cryptosporidium* and *Giardia* in aqueous matrices. EPA Method 1623 requires filtration, immunomagnetic separation of the oocysts and cysts from the material captured, and enumeration of the target organisms based on the results of immunofluorescence assay, 4',6-diamidino-2-phenylindole (DAPI) staining results, and differential interference contrast microscopy.

The interlaboratory validation of EPA Method 1623 conducted by EPA used the Pall Gelman capsule filtration procedure, Dynal immunomagnetic separation (IMS) procedure, and Meridian sample staining procedure described in this document. Alternate procedures are allowed, provided that required quality control tests are performed and all quality control acceptance criteria in this method are met.

Since the interlaboratory validation of EPA Method 1623, interlaboratory validation studies have been performed to demonstrate the equivalency of modified versions of the method using the following components:

- Whatman Nuclepore CryptTest™ filter (no longer available)
- IDEXX Filta-Max™ filter
- Pall Gelman Envirochek™ HV filter
- Waterborne Aqua-Glo™ G/C Direct FL antibody stain
- Waterborne Crypt-a-Glo™ and Giardi-a-Glo™ antibody stains
- BTF EasySeed™ irradiated oocysts and cysts for use in routine QC samples

The validation studies for these modified versions of the method met EPA's performance-based measurement system Tier 2 validation for nationwide use (see Section 9.1.2 for details), and have been accepted by EPA as equivalent in performance to the original version of the method validated by EPA. The equipment and reagents used in these modified versions of the method are noted in Sections 6 and 7 of the method; ~~the procedures for using these equipment and reagent options are available from the manufacturers.~~

Because this is a performance-based method, other alternative components not listed in the method may be available for evaluation and use by the laboratory. Confirming the acceptable performance of a modified version of the method using alternate components in a single laboratory does not require that an interlaboratory validation study be conducted. However, method modifications validated only in a single laboratory have not undergone sufficient testing to merit inclusion in the method. Only those modified versions of the method that have been demonstrated as equivalent at multiple laboratories on multiple water sources through a Tier 2 interlaboratory study will be cited in the method.

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Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA

1.0 Scope and Application

- 1.1 This method is for determination of the identity and concentration of *Cryptosporidium* (CAS Registry number 137259-50-8) and *Giardia* (CAS Registry number 137259-49-5) in water by ~~filtration-concentration~~, immunomagnetic separation (IMS), and immunofluorescence assay (FA) microscopy. *Cryptosporidium* and *Giardia* may be confirmed using 4',6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy. The method has been validated in surface water, but may be used in other waters, provided the laboratory demonstrates that the method's performance acceptance criteria are met.
- 1.2 This method is designed to meet the survey and monitoring requirements of the U.S. Environmental Protection Agency (EPA). It is based on laboratory testing of recommendations by a panel of experts convened by EPA. The panel was charged with recommending an improved protocol for recovery and detection of protozoa that could be tested and implemented with minimal additional research.
- 1.3 This method will not identify the species of *Cryptosporidium* or *Giardia* or the host species of origin, nor can it determine the viability or infectivity of detected oocysts and cysts.
- 1.4 This method is for use only by persons experienced in the determination of *Cryptosporidium* and *Giardia* by filtration, IMS, and FA. Experienced persons are defined in Section 22.2 as analysts ~~(and principal analysts, in applicable programs)~~. Laboratories unfamiliar with analyses of environmental samples by the techniques in this method should gain experience using water filtration techniques, IMS, fluorescent antibody staining with monoclonal antibodies, and microscopic examination of biological particulates using bright-field and DIC microscopy.
- 1.5 Any modification of the method beyond those expressly permitted is subject to the application and approval of alternative test procedures under 40 *CFR* Part 141.27.

2.0 Summary of Method

- 2.1 A water sample is filtered and the oocysts, cysts, and extraneous materials are retained on the filter. Although EPA has only validated the method using laboratory filtration of bulk water samples shipped from the field, field-filtration also ~~can may~~ be used.
- 2.2 Elution and separation
 - 2.2.1 Materials on the filter are eluted and the eluate is centrifuged to pellet the oocysts and cysts, and the supernatant fluid is aspirated.
 - 2.2.2 The oocysts and cysts are magnetized by attachment of magnetic beads conjugated to anti-*Cryptosporidium* and anti-*Giardia* antibodies. The magnetized oocysts and cysts are separated from the extraneous materials using a magnet, and the extraneous materials are discarded. The magnetic bead complex is then detached from the oocysts and cysts.
- 2.3 Enumeration
 - 2.3.1 The oocysts and cysts are stained on well slides with fluorescently labeled monoclonal antibodies and 4',6-diamidino-2-phenylindole (DAPI). The stained sample is examined using fluorescence and differential interference contrast (DIC) microscopy.
 - 2.3.2 Qualitative analysis is performed by scanning each slide well for objects that meet the size, shape, and fluorescence characteristics of *Cryptosporidium* oocysts or *Giardia* cysts. ~~Potential oocysts or cysts are confirmed through DAPI staining characteristics and~~

~~DIC microscopy. Oocysts and cysts are identified when the size, shape, color, and morphology agree with specified criteria and examples in a photographic library.~~

- 2.3.3** Quantitative analysis is performed by counting the total number of objects on the slide confirmed as oocysts or cysts.
- 2.4** Quality is assured through reproducible calibration and testing of the filtration, immunomagnetic separation (IMS), staining, and microscopy systems. Detailed information on these tests is provided in Section 9.0.

3.0 Definitions

- 3.1** *Cryptosporidium* is ~~defined as~~ a protozoan parasite potentially found in water and other media. The six species of *Cryptosporidium* and their potential hosts are *C. parvum* (mammals, including humans); *C. baileyi* and *C. meleagridis* (birds); *C. muris* (rodents); *C. serpentis* (reptiles); and *C. nasorum* (fish). *Cryptosporidium* is defined in this method as those determined by brilliant apple green fluorescence under UV light, size (4 to 6 μm), and shape (round to oval), excluding atypical organisms specifically identified as other microbial organisms by FITC and DIC (for example, those possessing spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.).
- 3.2** *Giardia* is a protozoan parasite potentially found in water and other media. The two species of *Giardia* and their potential hosts are *G. intestinalis* (humans) and *G. muris* (mice). *Giardia* is defined in this method as those determined by brilliant apple green fluorescence under UV light, size (8 to 18 μm long by 5 to 15 μm wide), and shape (oval), excluding atypical organisms specifically identified as other microbial organisms by FITC and DIC (for example, those possessing spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.).
- 3.3** Definitions for other terms used in this method are given in the glossary (Section 22.0).

4.0 Contamination, Interferences, and Organism Degradation

- 4.1** Turbidity caused by inorganic and organic debris can interfere with the concentration, separation, and examination of the sample for *Cryptosporidium* oocysts and *Giardia* cysts. In addition to naturally-occurring debris, such as clays and algae, chemicals, such as iron and alum coagulants and polymers, ~~may be~~ added to finished waters during the treatment process, ~~which~~ may result in additional interference.
- 4.2** Organisms and debris that autofluoresce or demonstrate non-specific fluorescence, such as algal and yeast cells, when examined by epifluorescent microscopy, may interfere with the detection of oocysts and cysts and contribute to false positives by immunofluorescence assay (FA) (Reference 20.1).
- 4.3** Solvents, reagents, labware, and other sample-processing hardware may yield artifacts that may cause misinterpretation of microscopic examinations for oocysts and cysts. All materials used shall be demonstrated to be free from interferences under the conditions of analysis by running a method blank (negative control sample) initially and a minimum of every week or after changes in source of reagent water. Specific selection of reagents and purification of solvents and other materials may be required.
- ~~**4.4** Interferences co-extracted from samples will vary considerably from source to source, depending on the water being sampled. Experience suggests that high levels of algae, bacteria, and other protozoa can interfere in the identification of oocysts and cysts (Reference 20.1).~~

- 4.4** Freezing samples, filters, eluates, concentrates, or slides may interfere with the detection and/or identification of oocysts and cysts.
- 4.5** All equipment should be cleaned according to manufacturers' instructions. Disposable supplies should be used wherever possible.

5.0 Safety

- 5.1** The biohazard associated with, and the risk of infection from, oocysts and cysts is high in this method because live organisms are handled. This method does not purport to address all of the safety problems associated with its use. It is the responsibility of the laboratory to establish appropriate safety and health practices prior to use of this method. In particular, laboratory staff must know and observe the safety procedures required in a microbiology laboratory that handles pathogenic organisms while preparing, using, and disposing of sample concentrates, reagents and materials, and while operating sterilization equipment.
- 5.2** The toxicity or carcinogenicity of each compound or reagent used in this method has not been precisely determined; however, each chemical compound should be treated as a potential health hazard. Exposure to these compounds should be reduced to the lowest possible level. The laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration regulations regarding the safe handling of the chemicals specified in this method. A reference file of material safety data sheets should be made available to all personnel involved in these analyses. Additional information on laboratory safety can be found in References 20.2 through 20.5.
- 5.3** Samples may contain high concentrations of biohazards and toxic compounds, and must be handled with gloves and opened in a biological safety cabinet to prevent exposure. Reference materials and standards containing oocysts and cysts must also be handled with gloves and laboratory staff must never place gloves in or near the face after exposure to solutions known or suspected to contain oocysts and cysts. Do not mouth-pipette.
- 5.4** Laboratory personnel must change gloves after handling filters and other contaminant-prone equipment and reagents. Gloves must be removed or changed before touching any other laboratory surfaces or equipment.
- 5.5** Centers for Disease Control (CDC) regulations (42 CFR 72) prohibit interstate shipment of more than 4 L of solution known to contain infectious materials ([see http://www.cdc.gov/od/ohs/biosfty/shipregs.htm for details](http://www.cdc.gov/od/ohs/biosfty/shipregs.htm)). State regulations may contain similar regulations for intrastate commerce. Unless the sample is known or suspected to contain *Cryptosporidium*, *Giardia*, or other infectious agents (e.g., during an outbreak), samples should be shipped as noninfectious and should not be marked as infectious. If a sample is known or suspected to be infectious, and the sample must be shipped to a laboratory by a transportation means affected by CDC or state regulations, the sample should be shipped in accordance with these regulations.

6.0 Equipment and Supplies

NOTE: Brand names, suppliers, and part numbers are for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using apparatus and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.

- 6.1** Sample collection equipment for shipment of bulk water samples for laboratory filtration. Collapsible LDPE cubitainer for collection of 10-L bulk sample(s)—Cole Parmer cat. no. U-06100-30 or equivalent. Fill completely to ensure collection of a full 10-L sample. Discard after one use.
- 6.2** Equipment for sample filtration. Three options have been demonstrated to be acceptable for use with Method 1623. Other options may be used if their acceptability is demonstrated according to the procedures outlined in Section 9.1.2.
 - 6.2.1** Cubitainer spigot to facilitate laboratory filtration of sample (for use with any filtration option)—Cole Parmer cat. no. U-06061-01, or equivalent.
 - 6.2.2** **Original** Envirochek™ sampling capsule **or Envirochek™ HV sampling capsule** equipment requirements (for use with the procedure described in Section 12.2). The versions of the method using ~~this filter~~ **were these filters** ~~were~~ validated using 10-L **and 50-L** sample volumes, **respectively**. Alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and source water samples (Section 9.1.2).
 - 6.2.2.1** Sampling capsule
 - 6.2.2.1.1** Envirochek™, Pall Gelman Laboratory, Ann Arbor, MI, product no. 12110 (**individual filter**) **and or product** no.12107 (box of 25 filters) (www.pall.com/gelman or (800) 521-1520 ext. 2)
 - 6.2.2.1.2** Envirochek™ HV, Pall Gelman Laboratory, Ann Arbor, MI, product no. 12099 (individual filter) or product no.12098 (box of 25 filters) (www.pall.com/gelman or (800) 521-1520 ext. 2)
 - 6.2.2.2** Laboratory shaker with arms for agitation of sampling capsules
 - 6.2.2.2.1** Laboratory shaker—Lab-Line model 3589, VWR Scientific cat. no. 57039-055, Fisher cat. no. 14260-11, or equivalent
 - 6.2.2.2.2** Side arms for laboratory shaker—Lab-Line Model 3587-4, VWR Scientific cat. no. 57039-045, Fisher cat. no. 14260-13, or equivalent
 - ~~**6.2.3** CrypTest™ capsule filter equipment requirements. Follow the manufacturer's instructions when using this filtration option. The version of the method using this filter was validated using 10-L sample volumes; alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and matrix samples (Section 9.1.2).~~
 - ~~**6.2.3.1** Capsule filter—CrypTest™, Whatman Inc, Clifton, NJ, product no. 610064~~
 - ~~**6.2.3.2** Cartridge housing—Ametek 5-in. clear polycarbonate, Whatman cat. no. 71503, or equivalent~~
 - ~~**6.2.3.3** Ultrasonic bath—VWR Model 75T#21811-808, or equivalent~~
 - ~~**6.2.3.4** Laboratory tubing—Tygon formula R-3603, or equivalent~~
 - 6.2.3** Filtamax™ foam filter equipment requirements (for use with the procedure described in Section 12.3). The version of the method using this filter was validated using 50-L

sample volumes; alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and matrix samples (Section 9.1.2).

- 6.2.3.1** Foam filter—Filta-Max™, IDEXX, Westbrook, ME. Filter module and membrane: product code FMC 10601; filter membranes (100 pack), product code FMC 10800

NOTE: Check at least one filter per batch to ensure that the filters have not been affected by improper storage or other factors that could result in brittleness or other problems. At a minimum confirm that the test filter expands properly in water before using the batch or shipping filters to the field.

- 6.2.3.2** Filter processing equipment—Filta-Max starter kit, IDEXX, Westbrook, ME, cat. no. FMC 11002. Includes all equipment required to run and process Filta-Max filter modules (manual wash station (FMC 10102) including plunger head (FMC 12001), elution tubing set (FMC 10301), vacuum set (FMC 10401), filter housing (FMC 10501), and magnetic stirrer (FMC 10901).

6.3 Ancillary sampling equipment

- 6.3.1** Tubing—Glass, polytetrafluoroethylene (PTFE), high-density polyethylene (HDPE), or other tubing to which oocysts and cysts will not easily adhere—Tygon formula R-3603, or equivalent. If rigid tubing (glass, PTFE, HDPE) is used and the sampling system uses a peristaltic pump, a minimum length of compressible tubing may be used in the pump. Before use, the tubing must be autoclaved, thoroughly rinsed with detergent solution, followed by repeated rinsing with reagent water to minimize sample contamination. Alternately, decontaminate using hypochlorite solution, sodium thiosulfate, and multiple reagent water rinses; dispose of tubing when wear is evident. Dispose of tubing after one use whenever possible.
- 6.3.2** Flow control valve—0.5 gpm (0.03 L/s), Bertram Controls, Plast-O-Matic cat. no. FC050B½-PV, or equivalent; or 0.4- to 4-Lpm flow meter with valve—Alamo Water Treatment, San Antonio, TX, cat. no. R5310, or equivalent
- 6.3.3** Centrifugal pump—~~Grainger, Springfield, VA, cat. no. 2P613~~ Simer, model number, M40, or equivalent.
- 6.3.4** Flow meter—Sameco cold water totalizer, E. Clark and Associates, Northboro, MA, product no. WFU 10.110, or equivalent

6.4 Equipment for spiking samples in the laboratory

- 6.4.1** 10-L carboy with bottom delivery port (½")—Cole-Palmer cat. no. 06080-42, or equivalent; calibrate to 10.0 L and mark level with waterproof marker
- 6.4.2** Stir bar—Fisher cat. no. 14-511-93, or equivalent
- 6.4.3** Stir plate—Fisher cat. no. 14-493-120S, or equivalent
- 6.4.4** Hemacytometer—Neubauer type, Hauser Scientific, Horsham, PA, cat. no. 3200 or 1475, or equivalent
- 6.4.5** Hemacytometer coverslip—Hauser Scientific, cat. no. 5000 (for hemacytometer cat. no. 3200) or 1461 (for hemacytometer cat. no. 1475), or equivalent
- 6.4.6** Lens paper without silicone—Fisher cat. no. 11-995, or equivalent
- 6.4.7** Polystyrene or polypropylene conical tubes with screw caps—15- and 50-mL

- 6.4.8** Equipment required for enumeration of spiking suspensions using membrane filters
 - 6.4.8.1** Glass microanalysis filter holder—25-mm-diameter, with fritted glass support, Fisher cat. no. 09-753E, or equivalent. Replace stopper with size 8, one-hole rubber stopper, Fisher Cat. No. 14-135M, or equivalent.
 - 6.4.8.2** Three-port vacuum filtration manifold and vacuum source—Fisher Cat. No. 09-753-39A, or equivalent
 - 6.4.8.3** Cellulose acetate support membrane—1.2- μ m-pore-size, 25-mm-diameter, Fisher cat. no. A12SP02500, or equivalent
 - 6.4.8.4** Polycarbonate track-etch hydrophilic membrane filter—1- μ m-pore-size, 25-mm-diameter, Fisher cat. no. K10CP02500, or equivalent
 - 6.4.8.5** 100 \times 15 mm polystyrene petri dishes (bottoms only)
 - 6.4.8.6** 60 \times 15 mm polystyrene petri dishes
 - 6.4.8.7** Glass microscope slides—1 in. \times 3 in or 2 in. \times 3 in.
 - 6.4.8.8** Coverslips—25 mm²
- 6.5** Immunomagnetic separation (IMS) apparatus
 - 6.5.1** Sample mixer—Dynal Inc., Lake Success, NY, cat. no. 947.01, or equivalent
 - 6.5.2** Magnetic particle concentrator for 10-mL test tubes—Dynal MPC-1® , cat. no. 120.01, or equivalent
 - 6.5.3** Magnetic particle concentrator for microcentrifuge tubes—Dynal MPC-M®, cat. no. 120.09, or equivalent
 - 6.5.4** Flat-sided sample tubes—16 \times 125 mm Leighton-type tubes with 60 \times 10 mm flat-sided magnetic capture area, Dynal L10, cat. no. 740.03, or equivalent
- 6.6** Powder-free latex gloves—Fisher cat no. 113945B, or equivalent
- 6.7** Graduated cylinders, autoclavable—10-, 100-, and 1000-mL
- 6.8** Centrifuges
 - 6.8.1** Centrifuge capable of accepting 15- to 250-mL conical centrifuge tubes and achieving 1500 \times G—International Equipment Company, Needham Heights, MA, Centrifuge Size 2, Model K with swinging bucket, or equivalent
 - 6.8.2** Centrifuge tubes—Conical, graduated, 1.5-, 50-, and 250-mL
- 6.9** Microscope
 - 6.9.1** Epifluorescence/differential interference contrast (DIC) with stage and ocular micrometers and 20X (N.A.=0.4) to 100X (N.A.=1.3) objectives—Zeiss™ Axioskop, Olympus™ BH, or equivalent
 - 6.9.2** Excitation/band-pass filters for immunofluorescence assay (FA)—Zeiss™ 487909 or equivalent, including, 450- to 490-nm exciter filter, 510-nm dichroic beam-splitting mirror, and 515- to 520-nm barrier or suppression filter

6.9.3 Excitation/band-pass filters for DAPI—Filters cited below (Chroma Technology, Brattleboro, VT), or equivalent

Microscope model	Fluoro-chrome	Excitation filter (nm)	Dichroic beam-splitting mirror (nm)	Barrier or suppression filter (nm)	Chroma catalog number
Zeiss™ -Axioskop	DAPI (UV)	340-380	400	420	CZ902
Zeiss™ -IM35	DAPI (UV)	340-380	400	420	CZ702
Olympus™ BH	DAPI (UV)	340-380	400	420	11000
	Filter holder				91002
Olympus™ BX	DAPI (UV)	340-380	400	420	11000
	Filter holder				91008
Olympus™ IMT2	DAPI (UV)	340-380	400	420	11000
	Filter holder				91003

6.10 Ancillary equipment for microscopy

6.10.1 Well slides— Spot-On well slides, Dynal cat. no. 740.04; treated, 12-mm diameter well slides, Meridian Diagnostics Inc., Cincinnati, OH, cat. no. R2206; or equivalent

6.10.2 Glass coverslips—22 × 50 mm

6.10.3 Nonfluorescing immersion oil—Type FF, Cargille cat. no. 16212, or equivalent

6.10.4 Micropipette, adjustable: 0- to 10-μL with 0- to 10-μL tips
10- to 100-μL, with 10- to 200-μL tips
100- to 1000-μL with 100- to 1000-μL tips

6.10.5 Forceps—Splinter, fine tip

6.10.6 Forceps—Blunt-end

6.10.7 Desiccant—Drierite™ Absorbent, Fisher cat. no. 07-577-1A, or equivalent

6.10.8 Humid chamber—A tightly sealed plastic container containing damp paper towels on top of which the slides are placed

6.11 Pipettes—Glass or plastic

6.11.1 5-, 10-, and 25-mL

6.11.2 Pasteur, disposable

6.12 Balances

6.12.1 Analytical—Capable of weighing 0.1 mg

6.12.2 Top loading—Capable of weighing 10 mg

6.13 pH meter

6.14 Incubator—Fisher Scientific Isotemp™, or equivalent

6.15 Vortex mixer—Fisons Whirlmixer, or equivalent

6.16 Vacuum source—Capable of maintaining 25 in. Hg, equipped with shutoff valve and vacuum gauge

6.17 Miscellaneous labware and supplies

6.17.1 Test tubes and rack

6.17.2 Flasks—Suction, Erlenmeyer, and volumetric, various sizes

6.17.3 Beakers—Glass or plastic, 5-, 10-, 50-, 100-, 500-, 1000-, and 2000-mL

6.17.4 Lint-free tissues

- 6.18** 10- to 15-L graduated container—Fisher cat. no. 02-961-50B, or equivalent; calibrate to 9.0, 9.5, 10.0, 10.5, and 11.0 L and mark levels with waterproof marker
- 6.19** Filters for filter-sterilizing reagents—Sterile Acrodisc, 0.45 μm , Gelman Sciences cat no. 4184, or equivalent

7.0 Reagents and Standards

7.1 Reagents for adjusting pH

- 7.1.1** Sodium hydroxide (NaOH)—ACS reagent grade, 6.0 N and 1.0 N in reagent water
- 7.1.2** Hydrochloric acid (HCl)—ACS reagent grade, 6.0 N, 1.0 N, and 0.1 N in reagent water

NOTE: Due to the low volumes of pH-adjusting reagents used in this method, and the impact that changes in pH have on the immunofluorescence assay, the laboratory should purchase standards at the required normality directly from a vendor. Normality should not be adjusted by the laboratory.

- 7.2** Solvents—Acetone, glycerol, ethanol, and methanol, ACS reagent grade
- 7.3** Reagent water—Water in which oocysts and cysts and interfering materials and substances, including magnetic minerals, are not detected by this method
- 7.4** Reagents for eluting filters

NOTE: Laboratories should store prepared eluting solution for no more than 1 week or when noticeably turbid, whichever comes sooner.

7.4.1 Reagents for eluting Envirochek™ sampling capsules (Section 6.2.2)

- 7.4.1.1** Laureth-12—PPG Industries, Gurnee, IL, cat. no. 06194, or equivalent. Store Laureth-12 as a 10% solution in reagent water. Weigh 10 g of Laureth-12 and dissolve using a microwave or hot plate in 90 mL of reagent water. Dispense 10-mL aliquots into sterile vials and store at room temperature for up to 2 months, or in the freezer for up to a year.
- 7.4.1.2** 1 M Tris, pH 7.4—Dissolve 121.1 g Tris (Fisher cat. no. BP152) in 700 mL of reagent water and adjust pH to 7.4 with 1 N HCl or NaOH. Dilute to a final 1000 mL with reagent water and adjust the final pH. Filter-sterilize through a 0.2- μm membrane into a sterile plastic container and store at room temperature.
- 7.4.1.3** 0.5 M EDTA, 2 Na, pH 8.0—Dissolve 186.1 g ethylenediamine tetraacetic acid, disodium salt dihydrate (Fisher cat. no. S311) in 800 mL of reagent water and adjust pH to 8.0 with 6.0 N HCl or NaOH. Dilute to a final volume of 1000 mL with reagent water and adjust to pH 8.0 with 1.0 N HCl or NaOH.
- 7.4.1.4** Antifoam A—Sigma Chemical Co. cat. no. A5758, or equivalent
- 7.4.1.5** Preparation of elution buffer solution—Add the contents of a pre-prepared Laureth-12 vial (Section 7.4.1.1) to a 1000-mL graduated cylinder. Rinse the vial several times to ensure the transfer of the detergent to the cylinder. Add 10 mL of Tris solution (Section 7.4.1.2), 2 mL of EDTA solution (Section 7.4.1.3), and 150 μL Antifoam A (Section 7.4.1.4). Dilute to 1000 mL with reagent water.

7.4.2 Reagents for eluting CrypTest™ capsule filters (Section 6.2.3). To 900 mL of reagent water add 8.0 g NaCl, 0.2 g KH_2PO_4 , 2.9 g $\text{Na}_2\text{HPO}_4 \cdot (12\text{H}_2\text{O})$, 0.2 g KCl, 0.2 g sodium lauryl sulfate (SDS), 0.2 mL Tween 80, and 0.02 mL Antifoam A (Sigma Chemical Co.

cat. no. A5758, or equivalent). Adjust volume to 1 L with reagent water and adjust pH to 7.4 with 1 N NaOH or HCl.

- 7.4.2** Reagents for eluting Filta-Max™ foam filters (Section 6.2.3)
- 7.4.2.1** Phosphate buffered saline (PBS), pH 7.4—Sigma Chemical Co. cat. no. P-3813, or equivalent. Alternately, prepare PBS by adding the following to 1 L of reagent water: 8 g NaCl; 0.2 g KCl; 1.15 g Na₂HPO₄, anhydrous; and 0.2 g KH₂PO₄.
- 7.4.2.2** Tween 20—Sigma Chemical Co. cat. no. P-7949, or equivalent
- 7.4.2.3** High-vacuum grease—BDH/Merck. cat. no. 636082B, or equivalent
- 7.4.2.4** Preparation of PBST elution buffer. Add 100 µL of Tween 20 to prepared PBS (Section 7.4.3.1-7.4.2.1). Alternatively, add the contents of one sachet packet of PBS to 1.0 L of reagent water. Dissolve by stirring for 30 minutes. Add 100 µL of Tween 20. Mix by stirring for 5 minutes.
- 7.5** Reagents for immunomagnetic separation (IMS)—Dynabeads® GC-Combo, Dynal cat. nos. 730.02, 730.12, or equivalent
- 7.6** Direct antibody labeling reagents for detection of oocysts and cysts. Store reagents at 0°C to 8°C and return promptly to this temperature after each use. Do not allow any of the reagents to freeze. The reagents should be protected from exposure to light. Diluted, unused working reagents should be discarded after 48 hours. Discard reagents after the expiration date is reached. The labeling reagents in Sections 7.6.1-7.6.3 have been approved for use with this method.
- 7.6.1** Merifluor *Cryptosporidium*/*Giardia*, Meridian Diagnostics cat. no. 250050, Cincinnati, OH, or equivalent
- 7.6.2** Aqua-Glo™ G/C Direct FL, Waterborne cat. no. A100FLR, New Orleans, LA, or equivalent
- 7.6.3** Crypt-a-Glo™ and Giardi-a-Glo™, Waterborne cat. nos. A400FLR and A300FLR, respectively, New Orleans, LA, or equivalent

NOTE: *If a laboratory will use multiple types of labeling reagents, the laboratory must demonstrate acceptable performance through an initial precision and recovery test (Section 9.4) for each type, and must perform positive and negative staining controls for each batch of slides stained using each product. However, the laboratory is not required to analyze additional ongoing precision and recovery samples or method blank samples for each type. The performance of each labeling reagent used also should be monitored in each source water type.*

- 7.6.4** Diluent for labeling reagents—Phosphate buffered saline (PBS) (Section 7.4.1). pH 7.4—Sigma Chemical Co. cat. no. P-3813, or equivalent. Alternately, prepare PBS by adding the following to 1 L of reagent water: 8 g NaCl; 0.2 g KCl; 1.15 g Na₂HPO₄, anhydrous; and 0.2 g KH₂PO₄. Filter-sterilize (Section 6.19) or autoclave. Discard if growth is detected or after 6 months, whichever comes first.
- 7.7** 4',6-diamidino-2-phenylindole (DAPI) stain—Sigma Chemical Co. cat. no. D9542, or equivalent
- 7.7.1** Stock solution—Dissolve 2 mg/mL DAPI in absolute methanol. Prepare volume consistent with minimum use. Store at 0°C to 8°C in the dark. Do not allow to freeze. Discard unused solution when positive staining control fails.
- 7.7.2** Staining solution (1/5000 dilution in PBS [Section 7.6.4])—Add 10 µL of 2 mg/mL DAPI stock solution to 50 mL of PBS. Prepare daily. Store at 0°C to 8°C in the dark except when staining. Do not allow to freeze. The solution concentration may be increased up to 1 µg/mL if fading/diffusion of DAPI staining is encountered, but the staining solution must be tested first on expendable environmental samples to confirm that staining intensity is appropriate.

7.8 Mounting medium

7.8.1 DABCO/glycerol mounting medium (2%)—Dissolve 2 g of DABCO (Sigma Chemical Co. cat no. D-2522, or equivalent) in 95 mL of warm glycerol/PBS (60% glycerol, 40% PBS [Section 7.6.4]). After the DABCO has dissolved completely, adjust the solution volume to 100 mL by adding an appropriate volume of glycerol/PBS solution. Alternately, dissolve the DABCO in 40 mL of PBS, then add azide (1 mL of 100X, or 10% solution), then 60 mL of glycerol.

7.8.2 Mounting medium supplied with Merifluor direct labeling kit (Section 7.6.1)

7.9 Clear fingernail polish or clear fixative, PGC Scientifics, Gaithersburg, MD, cat. no. 60-4890, or equivalent

7.10 Oocyst and cyst suspensions for spiking

7.10.1 Enumerated spiking suspensions prepared by flow cytometer—not heat-fixed or formalin fixed: ~~Wisconsin State Laboratory of Hygiene Flow Cytometry Unit or equivalent.~~

7.10.1.1 Live, flow cytometer-sorted oocysts and cysts—Wisconsin State Laboratory of Hygiene Flow Cytometry Unit ([608] 224-6260), or equivalent

7.10.1.2 Irradiated, flow cytometer-sorted oocysts and cysts—flow cytometer-sorted oocysts and cysts—BTF EasySeed™ (www.biotechnologyfrontiers.com), or equivalent

7.10.2 Materials for manual enumeration of spiking suspensions

7.10.2.1 Purified *Cryptosporidium* oocyst stock suspension for manual enumeration—not heat-fixed or formalin-fixed: Sterling Parasitology Laboratory, University of Arizona, Tucson, or equivalent

7.10.2.2 Purified *Giardia* cyst stock suspension for manual enumeration—not heat-fixed or formalin-fixed: Waterborne, Inc., New Orleans, LA; Hyperion Research, Medicine Hat, Alberta, Canada; or equivalent

7.10.2.3 Tween-20, 0.01%—Dissolve 1.0 mL of a 10% solution of Tween-20 in 1 L of reagent water

7.10.3 Storage procedure—Store oocyst and cyst suspensions at 0°C to 810°C, until ready to use; do not allow to freeze

7.11 Additional reagents for enumeration of spiking suspensions using membrane filtration (Section 11.3.6)—Sigmacote® Sigma Company Product No. SL-2, or equivalent

8.0 Sample Collection and Storage

8.1 Sample collection, shipment, and receipt

8.1.1 **Sample collection.** Samples are collected as bulk samples and shipped to the laboratory for processing through the entire method, or are filtered in the field and shipped to the laboratory for processing from elution (Section 12.2.6) onward.

8.1.2 **Sample shipment.** Ambient water samples are dynamic environments and, depending on sample constituents and environmental conditions, *Cryptosporidium* oocysts or *Giardia* cysts present in a sample can degrade, potentially biasing analytical results. Samples that are not analyzed the same day they are collected should be chilled to reduce biological activity, and preserve the state of source water samples between collection and analysis. Samples ~~must~~ analyzed by an off-site laboratory should be shipped via overnight service on the day they are collected. ~~Overnight service may not be necessary if the samples are maintained below 10°C (but not frozen) and holding times are met.~~

NOTE: See transportation precautions in Section 5.5.

- 8.1.2.1** If samples are collected early in the day, chill samples as much as possible between collection and shipment by storing in a refrigerator or pre-icing the sample in a cooler. If the sample is pre-iced before shipping, replace with fresh ice immediately before shipment.
- 8.1.2.2** If samples are collected later in the day, these samples may be held overnight in a refrigerator. This should be considered for bulk water samples that will be shipped off-site, as this minimizes the potential for water samples collected during the summer to melt the ice in which they are packed and arrive at the laboratory at >10°C.
- 8.1.2.3** Samples should be shipped at ~~0°C to~~ < 10°C ~~8°C~~ and not frozen, unless the time required to chill the sample to ~~8°C~~ 10°C would prevent the sample from being shipped overnight for receipt at the laboratory the day after collection. Samples must not be allowed to freeze.
- 8.1.2.4** Public water systems shipping samples to off-site laboratories for analysis should include in the shipping container a means for monitoring the temperature of the sample during shipping to verify that the sample did not freeze or exceed 10°C. Suggested approaches for monitoring sample temperature during shipping are discussed in Section 8.1.4.
- 8.1.3** **Sample receipt.** Upon receipt, the laboratory must record the temperature. Samples that were not collected the same day they were received, and that are received at >10°C or frozen, or samples that the laboratory has determined exceeded >10°C or froze during shipment, must be rejected. After receipt, samples must be stored at the laboratory at < 10°C, and not frozen, until processed. ~~Results from samples shipped overnight to the laboratory and received at >8°C should be qualified by the laboratory..~~
- 8.1.4** **Suggestions on measuring sample temperature.** Given the importance of maintaining sample temperatures for *Cryptosporidium* and *Giardia* determination, laboratories performing analyses using this method must establish acceptance criteria for receipt of samples transported to their laboratory. Several options are available to measure sample temperature upon receipt at the laboratory and, in some cases, during shipment:
- 8.1.4.1** **Temperature sample.** One option, for filtered samples only (not for 10-L bulk samples), is for the sampler to fill a small, inexpensive sample bottle with water and pack this “temperature sample” next to the filtered sample. The temperature of this extra sample volume is measured upon receipt to estimate the temperature of the filter. Temperature sample bottles are not appropriate for use with bulk samples because of the potential effect that the difference in sample volume may have in temperature equilibration in the sample cooler. *Example product:* Cole Parmer cat. no. U-06252-20.
- 8.1.4.2** **Thermometer vial.** A similar option is to use a thermometer that is securely housed in a liquid-filled vial. Unlike temperature samples, the laboratory does not need to perform an additional step to monitor the temperature of the vial upon receipt, but instead just needs to read the thermometer. *Example product:* Eagle-Picher Sentry Temperature Vial 3TR-40CS-F or 3TR-40CS.
- 8.1.4.3** **iButton.** Another option for measuring the sample temperature during shipment and upon receipt is a Thermocron® iButton. An iButton is a small, waterproof device that contains a computer chip that can be programmed to record temperature at different time intervals. The information is then downloaded from the iButton onto a computer. The

iButton should be placed in a temperature sample (Section 8.1.4.1), rather than placed loose in the cooler. Information on Thermocron® iButtons is available from <http://www.ibutton.com/>. Distributors include <http://www.pointsix.com/>, <http://www.rdsdistributing.com>, and <http://www.scigiene.com/>.

8.1.4.4 Stick-on temperature strips. Another option is for the laboratory to apply a stick-on temperature strip to the outside of the sample container upon receipt at the laboratory. This option does not measure temperature as precisely as the other options, but still mitigates the risk of sample contamination while providing an indication of sample temperature to verify that the sample temperature is acceptable. *Example product:* Cole Parmer cat. no. U-90316-00.

8.1.4.5 Infrared thermometers. A final option is to measure the temperature of the surface of the sample container or filter using an infrared thermometer. The thermometer is pointed at the sample, and measures the temperature without coming in contact with the sample volume. *Example product:* Cole Parmer cat. no. EW-39641-00.

As with other laboratory equipment, all temperature measurement devices must be calibrated routinely to ensure accurate measurements. See the *EPA Manual for the Certification of Laboratories Analyzing Drinking Water* (Reference 20.6) for more information.

8.2 Sample holding times. Samples must be processed or examined within each of the holding times specified in Sections 8.2.1 - 8.2.4. Sample processing should be completed as soon as possible by the laboratory. The laboratory should complete sample filtration, elution, concentration, purification, and staining the day the sample is received wherever possible. However, the laboratory is permitted to split up the sample processing steps if processing a sample completely in one day is not possible. If this is necessary, sample processing can be halted after filtration, application of the purified sample onto the slide, or staining. Table 1, in Section 21.0 provides a breakdown of the holding times for each set of steps. Sections 8.2.1 through 8.2.4 provide descriptions of these holding times.

8.2.1 Sample collection and filtration. Sample elution must be initiated within 96 hours of sample collection (if shipped to the laboratory as a bulk sample) or filtration (if filtered in the field).

8.2.2 Sample elution, concentration, and purification. The laboratory must complete the elution, concentration, and purification (Sections 12.2.6 through 13.3.3.11) in one work day. It is critical that these steps be completed in one work day to minimize the time that any target organisms present in the sample sit in eluate or concentrated matrix. This process ends with the application of the purified sample on the slide for drying.

8.2.3 Staining. The sample must be stained within 72 hours of application of the purified sample to the slide.

8.2.4 Examination. Although immunofluorescence assay (FA) and 4',6-diamidino-2-phenylindole (DAPI) and differential interference contrast (DIC) microscopy examination and confirmation should be performed immediately after staining is complete, laboratories have up to 7 days from completion of sample staining to complete the examination and confirmation of samples. However, if fading/diffusion of FITC or DAPI staining is noticed, the laboratory must reduce this holding time. In addition the laboratory may adjust the concentration of the DAPI staining solution (Sections 7.7.2) so that fading/diffusion does not occur.

8.5 Spiking suspension enumeration holding times. Flow-cytometer-sorted spiking suspensions (Sections 7.10.1 and 11.2) used for spiked quality control (QC) samples (Section 9) must be used within the expiration date noted on the suspension. ~~Laboratories should use flow-cytometer-~~

~~sorted spiking suspensions containing live organisms within two weeks of preparation at the flow cytometry laboratory.~~ Manually enumerated spiking suspensions must be used within 24 hours of enumeration of the spiking suspension if the hemacytometer chamber technique is used (Section 11.3.4); or within 24 hours of application of the spiking suspension to the slides if the well slide or membrane filter enumeration technique is used (Sections 11.3.5 and 11.3.6). **Oocyst and cyst suspensions must be stored at 0°C to 10°C, until ready to use; do not allow to freeze**

9.0 Quality Control

9.1 Each laboratory that uses this method is required to operate a formal quality assurance (QA) program (Reference 20.7). The minimum requirements of this program consist of an initial demonstration of laboratory capability through performance of the initial precision and recovery (IPR) test (Section 9.4), analysis of spiked samples to evaluate and document data quality, and analysis of standards and blanks as tests of continued performance. Laboratory performance is compared to established performance criteria to determine if the results of analyses meet the performance characteristics of the method.

9.1.1 A test of the microscope used for detection of oocysts and cysts is performed prior to examination of slides. This test is described in Section 10.0.

9.1.2 In recognition of advances that are occurring in analytical technology, the laboratory is permitted to modify certain method procedures to improve recovery or lower the costs of measurements, provided that all required quality control (QC) tests are performed and all QC acceptance criteria are met. Method procedures that can be modified include front-end techniques, such as filtration or immunomagnetic separation (IMS). The laboratory is not permitted to use an alternate determinative technique to replace immunofluorescence assay in this method (the use of different determinative techniques are considered to be different methods, rather than modified version of this method). However, the laboratory is permitted to modify the immunofluorescence assay procedure, provided that all required QC tests are performed (Section 9.1.2.1) and all QC acceptance criteria are met (see guidance on the use of multiple labeling reagents in Section 7.6).

NOTE: *Method modifications should be considered only to improve method performance, reduce cost, or reduce sample processing time. Method modifications that reduce cost or sample processing time, but that result in poorer method performance should not be used.*

9.1.2.1 Method modification validation/equivalency demonstration requirements

9.1.2.1.1 **Method modifications at a single laboratory.** Each time a modification is made to this method for use in single laboratory, the laboratory must, **at a minimum**, validate the modification according to Tier 1 of EPA's performance-based measurement system (PBMS) (Table 2 ~~and Reference 20.8~~) to demonstrate that the modification produces results equivalent or superior to results produced by this method as written. Briefly, each time a modification is made to this method, the laboratory is required to demonstrate acceptable modified method performance through the IPR test (Section 9.4). IPR results must meet the QC acceptance criteria in Tables 3 and 4 in Section 21.0, and should be comparable to previous results using the unmodified procedure. Although not required, the laboratory also should perform a matrix spike/matrix spike duplicate (MS/MSD) test to demonstrate the performance of the modified method in at least one real-world matrix before

analyzing field samples using the modified method. The laboratory is required to perform MS samples using the modified method at the frequency noted in Section 9.1.8. **If the modified method involves changes that cannot be adequately evaluated through these tests, additional tests may be required to demonstrate acceptability.**

9.1.2.1.2 Method modifications for nationwide approval. If the laboratory or a manufacturer seeks EPA approval of a method modification for nationwide use, the laboratory or manufacturer must, **at a minimum**, validate the modification according to Tier 2 of EPA's PBMS (Table 2 **and Reference 20.8**). Briefly, at least three laboratories must perform IPR tests (Section 9.4) and MS/MSD (Section 9.5) tests using the modified method, and all tests must meet the QC acceptance criteria specified in Tables 3 and 4 in Section 21.0. Upon nationwide approval, laboratories electing to use the modified method still must demonstrate acceptable performance in their own laboratory according to the requirements in Section 9.1.2.1.1. **If the modified method involves changes that cannot be adequately evaluated through these tests, additional tests may be required to demonstrate acceptability.**

9.1.2.2 The laboratory is required to maintain records of modifications made to this method. These records include the following, at a minimum:

9.1.2.2.1 The names, titles, addresses, and telephone numbers of the analyst(s) who performed the analyses and modification, and of the quality control officer who witnessed and will verify the analyses and modification.

9.1.2.2.2 A listing of the analyte(s) measured (*Cryptosporidium* and *Giardia*).

9.1.2.2.3 A narrative stating reason(s) for the modification.

9.1.2.2.4 Results from all QC tests comparing the modified method to this method, including:

- (a) IPR (Section 9.4)
- (b) MS/MSD (Section 9.5)
- (c) Analysis of method blanks (Section 9.6)

9.1.2.2.5 Data that will allow an independent reviewer to validate each determination by tracing the following processing and analysis steps leading to the final result:

- (a) Sample numbers and other identifiers
- (b) Source of spiking suspensions, as well as lot number and date received (Section 7.10)
- (c) Spike enumeration date and time
- (d) All spiking suspension enumeration counts and calculations (Section 11.0)
- (e) Sample spiking dates and times
- (f) Volume filtered (Section 12.2.5.2)
- (g) Filtration and elution dates and times
- (h) Pellet volume, resuspended concentrate volume, resuspended concentrate volume transferred to

- (i) IMS, and all calculations required to verify the percent of concentrate examined (Section 13.2)
 - (j) Purification completion dates and times (Section 13.3.3.11)
 - (k) Staining completion dates and times (Section 14.10)
 - (l) Staining control results (Section 15.2.1)
 - (m) All required examination information (Section 15.2.2)
 - (n) Examination completion dates and times (Section 15.2.4)
 - (o) Analysis sequence/run chronology
 - (p) Lot numbers of elution, IMS, and staining reagents
 - (q) Copies of bench sheets, logbooks, and other recordings of raw data
 - (r) Data system outputs, and other data to link the raw data to the results reported
- 9.1.3** The laboratory shall spike a separate sample aliquot from the same source to monitor method performance. This MS test is described in Section 9.5.1.
- 9.1.4** Analysis of method blanks is required to demonstrate freedom from contamination. The procedures and criteria for analysis of a method blank are described in Section 9.6.
- 9.1.5** The laboratory shall, on an ongoing basis, demonstrate through analysis of the ongoing precision and recovery (OPR) sample that the analysis system is in control. These procedures are described in Section 9.7.
- 9.1.6** The laboratory shall maintain records to define the quality of data that are generated. Development of accuracy statements is described in Sections 9.5.1.4 and 9.7.3.
- 9.1.7** The laboratory shall analyze one method blank (Section 9.6) and one OPR sample (Section 9.7) each week during which samples are analyzed if 20 or fewer field samples are analyzed during this period. The laboratory shall analyze one laboratory blank and one OPR sample for every 20 samples if more than 20 samples are analyzed in a week.
- 9.1.8** The laboratory shall analyze MS samples (Section 9.5.1) at a minimum frequency of 1 MS sample per 20 field samples and at least 12 months must elapse between the first and last MS sample. The laboratory should analyze an MS sample when samples are first received from a PWS for which the laboratory has never before analyzed samples to identify potential method performance issues with the matrix (Section 9.5.1; Tables 3 and 4). If an MS sample cannot be analyzed on the first sampling event, the first MS sample should be analyzed as soon as possible to identify potential method performance issues with the matrix.
- 9.2** Micropipette calibration
 - 9.2.1** Micropipettes must be sent to the manufacturer for calibration annually. Alternately, a qualified independent technician specializing in micropipette calibration can be used, or the calibration can be performed by the laboratory, provided the laboratory maintains a detailed procedure that can be evaluated by an independent auditor. Documentation on the precision of the recalibrated micropipette must be obtained from the manufacturer or technician.
 - 9.2.2** Internal and external calibration records must be kept on file in the laboratory's QA logbook.
 - 9.2.3** If a micropipette calibration problem is suspected, the laboratory shall tare an empty weighing boat on the analytical balance and pipette the following volumes of reagent water into the weigh boat using the pipette in question: 100% of the maximum

dispensing capacity of the micropipette, 50% of the capacity, and 10% of the capacity. Ten replicates should be performed at each weight. Record the weight of the water (assume that 1.00 mL of reagent water weighs 1.00 g) and calculate the relative standard deviation (RSD) for each. If the weight of the reagent water is within 1% of the desired weight (mL) and the RSD of the replicates at each weight is within 1%, then the pipette remains acceptable for use.

- 9.2.4** If the weight of the reagent water is outside the acceptable limits, consult the manufacturer's instruction manual troubleshooting section and repeat steps described in Section 9.2.3. If problems with the pipette persist, the laboratory must send the pipette to the manufacturer for recalibration.
- 9.3** Microscope adjustment and certification: Adjust the microscope as specified in Section 10.0. All of the requirements in Section 10.0 must be met prior to analysis of IPRs, blanks, OPRs, field samples, and MS/MSDs.
- 9.4** Initial precision and recovery (IPR)—To establish the ability to demonstrate control over the analytical system and to generate acceptable precision and recovery, the laboratory shall perform the following operations:
- 9.4.1** Using the spiking procedure in Section 11.4 and enumerated spiking suspensions (Section 7.10.1 or Section 11.3), spike, filter, elute, concentrate, separate (purify), stain, and examine four reagent water samples spiked with 100 to 500 oocysts and 100 to 500 cysts.
- 9.4.1.1** The laboratory is permitted to analyze the four spiked reagent samples on the same day or on as many as four different days (provided that the spiked reagent samples are analyzed consecutively), and also may use different analysts and/or reagent lots for each sample (however, the procedures used for all analyses must be identical). Laboratories should note that the variability of four measurements performed on multiple days or using multiple analysts or reagent lots may be greater than the variability of measurements performed on the same day with the same analysts and reagent lots. As a result, the laboratory is at a greater risk of generating unacceptably variable IPR results if the test is performed across multiple days, analysts, and /or reagent lots.
- 9.4.1.2** If more than one process will be used for filtration and/or separation of samples, a separate set of IPR samples must be prepared for each process.
- 9.4.1.3** The set of four IPR tests samples must be accompanied by analysis of an acceptable method blank (Section 9.6).
- 9.4.2** Using results of the four analyses, calculate the average percent recovery and the relative standard deviation (RSD) of the recoveries for *Cryptosporidium* and for *Giardia*. The RSD is the standard deviation divided by the mean, times 100.
- 9.4.3** Compare the RSD and the mean with the corresponding limits for initial precision and recovery in Tables 3 and 4 in Section 21.0. If the RSD and the mean meet the acceptance criteria, system performance is acceptable and analysis of blanks and samples may begin. If the RSD or the mean falls outside the range for recovery, system performance is unacceptable. In this event, trouble-shoot the problem by starting at the end of the method (see guidance in Section 7.9.5 9.7.5), correct the problem and repeat the test (Section 9.4.1).
- 9.5** Matrix spike (MS) and matrix spike duplicate (MSD):
- 9.5.1** Matrix spike—The laboratory shall spike and analyze a separate field sample aliquot to determine the effect of the matrix on the method's oocyst and cyst recovery. The MS and field sample must be collected from the same sampling location as split samples or as samples sequentially collected immediately after one another. The MS sample volume

analyzed must be within 10% of the field sample volume. The MS shall be analyzed according to the frequency in Section 9.1.8.

- 9.5.1.1** Analyze an unspiked field sample according to the procedures in Sections 12.0 to 15.0. Using the spiking procedure in Section 11.4 and enumerated spiking suspensions (Section 7.10.1 or Section 11.3), spike, filter, elute, concentrate, separate (purify), stain, and examine a second field sample aliquot with a similar ~~the~~ number of organisms as that used in the IPR or OPR tests (Sections 9.4 and 9.7).
- 9.5.1.2** For each organism, calculate the percent recovery (R) using the following equation.

$$R = 100 \times \frac{N_{sp} - N_s}{T}$$

where

R is the percent recovery

N_{sp} is the number of oocysts or cysts detected in the spiked sample

N_s is the number of oocysts or cysts detected in the unspiked sample

T is the true value of the oocysts or cysts spiked

- 9.5.1.3** Compare the recovery for each organism with the corresponding limits in Tables 3 and 4 in Section 21.0.

NOTE: Some sample matrices may prevent the acceptance criteria in Tables 3 and 4 from being met. An assessment of the distribution of MS recoveries across 430 MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 5.

- 9.5.1.4** As part of the QA program for the laboratory, method precision for samples should be assessed and records maintained. After the analysis of five samples ~~for which the spike recovery for each organism passes the tests in Section 9.5.1.3~~, the laboratory should calculate the average percent recovery (P) and the standard deviation of the percent recovery (s_r). Express the precision assessment as a percent recovery interval from $P - 2 s_r$ to $P + 2 s_r$ for each matrix. For example, if $P = 80\%$ and $s_r = 30\%$, the accuracy interval is expressed as 20% to 140%. The precision assessment should be updated regularly across all MS samples and stratified by MS samples for each source.
- 9.5.2** Matrix spike duplicate—MSD analysis is required as part of nationwide approval of a modified version of this method to demonstrate that the modified version of this method produces results equal or superior to results produced by the method as written (Section 9.1.2.1.2). At the same time the laboratory spikes and analyzes the second field sample aliquot in Section 9.5.1.1, the laboratory shall spike and analyze a third, identical field sample aliquot.

NOTE: Matrix spike duplicate samples are only required for Tier 2 validation studies. They are recommended for Tier 1 validation, but not required.

- 9.5.2.1** For each organism, calculate the percent recovery (R) using the equation in Section 9.5.1.2.
- 9.5.2.2** Calculate the mean of the number of oocysts or cysts in the MS and MSD (X_{mean}) ($= [MS + MSD]/2$).

- 9.5.2.3** Calculate the relative percent difference (RPD) of the recoveries using the following equation:

$$RPD = 100 \times \frac{|N_{MS} - N_{MSD}|}{X_{MEAN}}$$

where

RPD is the relative percent difference

N_{MS} is the number of oocysts or cysts detected in the MS

N_{MSD} is the number of oocysts or cysts detected in the MSD

X_{mean} is the mean number of oocysts or cysts detected in the MS and MSD

- 9.5.2.4** Compare the mean MS/MSD recovery and RPD with the corresponding limits in Tables 3 and 4 in Section 21.0 for each organism.

- 9.6** Method blank (negative control sample, laboratory blank): Reagent water blanks are analyzed to demonstrate freedom from contamination. Analyze the blank immediately ~~prior to~~ after analysis of the IPR test (Section 9.4) and OPR test (Section 9.7) and prior to analysis of samples for the week to demonstrate freedom from contamination.

- 9.6.1** Filter, elute, concentrate, separate (purify), stain, and examine at least one reagent water blank per week (Section 9.1.7) according to the procedures in Sections 12.0 to 15.0. If more than 20 samples are analyzed in a week, process and analyze one reagent water blank for every 20 samples.

- 9.6.2** Actions

- 9.6.2.1** If *Cryptosporidium* oocysts, *Giardia* cysts, or potentially interfering organisms or materials that may be misidentified as oocysts or cysts are not found in the method blank, the method blank test is acceptable and analysis of samples may proceed. ~~Any method blank in which oocysts or cysts are not detected is assumed to be uncontaminated and may be reported.~~

- 9.6.2.2** If *Cryptosporidium* oocysts, or *Giardia* cysts (as defined in Section 3) or any potentially interfering organism or materials that may be misidentified as oocysts or cysts are found in the method blank, the method blank test is unacceptable. Any field sample in a batch associated with an unacceptable method blank ~~a contaminated blank that shows the presence of one or more oocysts or cysts~~ is assumed to be contaminated and should be recollected, ~~if possible~~. Analysis of additional samples is halted until the source of contamination is eliminated, the method blank test is performed again, and no evidence of contamination is detected.

- 9.7** Ongoing precision and recovery (OPR; positive control sample; laboratory control sample): Using the spiking procedure in Section 11.4 and enumerated spiking suspensions (Section 7.10.1 or Section 11.3), filter, elute, concentrate, separate (purify), stain, and examine at least one reagent water sample spiked with 100 to 500 oocysts and 100 to 500 cysts each week to verify all performance criteria. The laboratory must analyze one OPR sample for every 20 samples if more than 20 samples are analyzed in a week. If multiple method variations are used, separate OPR samples must be prepared for each method variation. Adjustment and/or recalibration of the analytical system shall be performed until all performance criteria are met. Only after all performance criteria are met ~~may~~ should samples be analyzed.

- 9.7.1** Examine the slide from the OPR prior to analysis of samples from the same batch.

- 9.7.1.1** Using 200X to 400X magnification, more than 50% of the oocysts or cysts must appear undamaged and morphologically intact; otherwise, the

organisms in the spiking suspension may be of unacceptable quality or the analytical process ~~is~~ may be damaging the organisms. Examine the spiking suspension organisms directly (by centrifuging, if possible, to concentrate the organisms in a volume that can be applied directly to a slide). If the organisms appear undamaged and morphologically intact under DIC, determine the step or reagent that is causing damage to the organisms. Correct the problem and repeat the OPR test.

9.7.1.2 Identify and enumerate each organism using epifluorescence microscopy. The first three presumptive *Cryptosporidium* oocysts and three *Giardia* cysts identified in the OPR sample must be examined using FITC, DAPI, and DIC, as per Section 15.2, and the detailed characteristics (size, shape, DAPI category, and DIC category) reported on the *Cryptosporidium* and *Giardia* report form, as well as any additional comments on organism appearance, if notable.

9.7.2 For each organism, calculate the percent recovery (R) using the following equation:

$$R = 100 \times \frac{N}{T}$$

where:

R = the percent recovery

N = the number of oocysts or cysts detected

T = the number of oocysts or cysts spiked

9.7.3 Compare the recovery with the limits for ongoing precision and recovery in Tables 3 and 4 in Section 21.0.

9.7.4 Actions

9.7.4.1 If the recoveries for *Cryptosporidium* and *Giardia* meet the acceptance criteria, system performance is acceptable and analysis of ~~blanks and~~ samples may proceed.

9.7.4.2 If, ~~however,~~ the recovery for *Cryptosporidium* or *Giardia* falls outside of the ~~criteria range given,~~ system performance is unacceptable. Any sample in a batch associated with an unacceptable OPR sample is unacceptable. Analysis of additional samples is halted until the analytical system is brought under control. ~~In this event, there may be a problem with the microscope or with the filtration or separation systems.~~ Troubleshoot the problem using the procedures at Section 9.7.5 as a guide. After assessing the issue, perform another OPR test and verify that *Cryptosporidium* and *Giardia* recoveries meet the acceptance criteria. ~~All samples must be associated with an OPR that passes the criteria in Section 21.0. Samples that are not associated with an acceptable OPR must be flagged accordingly.~~

9.7.5 **Troubleshooting.** If an OPR sample has failed, and the cause of the failure is not known, the laboratory generally should identify the problem working backward in the analytical process from the microscopic examination to filtration.

9.7.5.1 **Quality of spiked organisms.** Examine the spiking suspension organisms directly (by centrifuging, if possible, to concentrate the organisms in a volume that can be applied directly to a slide). If the organisms appear damaged under DIC, obtain fresh spiking materials. If the organisms appear undamaged and morphologically intact, determine whether the problem is associated with the microscope system or antibody stain (Section 9.7.5.2).

9.7.5.2 Microscope system and antibody stain: To determine if the failure of the OPR test is due to changes in the microscope or problems with the antibody stain, re-examine the positive staining control (Section 15.2.1), check Köhler illumination, and check the fluorescence of the fluorescein-labeled monoclonal antibodies (Mabs) and 4',6-diamidino-2-phenylindole (DAPI). If results are unacceptable, re-examine the previously-prepared positive staining control to determine whether the problem is associated with the microscope or the antibody stain.

9.7.5.3 Separation (purification) system: To determine if the failure of the OPR test is attributable to the separation system, check system performance by spiking a 10-mL volume of reagent water with 100 - 500 oocysts and cysts and processing the sample through the IMS, staining, and examination procedures in Sections 13.3 through 15.0. **Recoveries should be greater than 70%.**

9.7.5.4 Filtration/elution/concentration system: If the failure of the OPR test is attributable to the filtration/elution/concentration system, check system performance by processing spiked reagent water according to the procedures in Section 12.2 through ~~13.2.2.1~~ **13.2.2**, and filter, stain, and examine the sample concentrate according to Section 11.3.6.

- 9.7.6** The laboratory should add results that pass the specifications in Section 9.7.3 to initial and previous ongoing data and update the QC chart to form a graphic representation of continued laboratory performance. The laboratory should develop a statement of laboratory accuracy (reagent water, raw surface water) by calculating the average percent recovery (R) and the standard deviation of percent recovery (s_r). Express the accuracy as a recovery interval from $R - 2 s_r$ to $R + 2 s_r$. For example, if $R = 95\%$ and $s_r = 25\%$, the accuracy is 45% to 145%.
- 9.8** The laboratory should periodically analyze an external QC sample, such as a performance evaluation or standard reference material, when available. The laboratory also should periodically participate in interlaboratory comparison studies using the method.
- 9.9** The specifications contained in this method can be met if the analytical system is under control. The standards used for initial (Section 9.4) and ongoing (Section 9.7) precision and recovery should be identical, so that the most precise results will be obtained. The microscope in particular will provide the most reproducible results if dedicated to the settings and conditions required for the determination of *Cryptosporidium* and *Giardia* by this method.
- 9.10** Depending on specific program requirements, field replicates may be collected to determine the precision of the sampling technique, and duplicate spiked samples may be required to determine the precision of the analysis.

10.0 Microscope Calibration and Analyst Verification

- 10.1** In a room capable of being darkened to near-complete darkness, assemble the microscope, all filters, and attachments. The microscope should be placed on a solid surface free from vibration. Adequate workspace should be provided on either side of the microscope for taking notes and placement of slides and ancillary materials.
- 10.2** Using the manuals provided with the microscope, all analysts must familiarize themselves with operation of the microscope.
- 10.3** Microscope adjustment and calibration (adapted from Reference 20.7)
- 10.3.1** Preparations for adjustment
- 10.3.1.1** The microscopy portion of this procedure depends upon proper alignment and adjustment of very sophisticated optics. Without proper alignment and adjustment, the microscope will not function at maximal

efficiency, and reliable identification and enumeration of oocysts and cysts will not be possible. Consequently, it is imperative that all portions of the microscope from the light sources to the oculars are properly adjusted.

- 10.3.1.2** While microscopes from various vendors are configured somewhat differently, they all operate on the same general physical principles. Therefore, slight deviations or adjustments may be required to make the procedures below work for a particular instrument.
- 10.3.1.3** The sections below assume that the mercury bulb has not exceeded time limits of operation, that the lamp socket is connected to the lamp house, and that the condenser is adjusted to produce Köhler illumination.
- 10.3.1.4** Persons with astigmatism should always wear contact lenses or glasses when using the microscope.

CAUTION: *In the procedures below, do not touch the quartz portion of the mercury bulb with your bare fingers. Finger oils can cause rapid degradation of the quartz and premature failure of the bulb.*

WARNING: *Never look at the ultraviolet (UV) light from the mercury lamp, lamp house, or the UV image without a barrier filter in place. UV radiation can cause serious eye damage.*

- 10.3.2** Epifluorescent mercury bulb adjustment: The purpose of this procedure is to ensure even field illumination. This procedure must be followed when the microscope is first used, when replacing bulbs, and if problems such as diminished fluorescence or uneven field illumination are experienced.
 - 10.3.2.1** Remove the diffuser lens between the lamp and microscope or swing it out of the transmitted light path.
 - 10.3.2.2** Using a prepared microscope slide, adjust the focus so the image in the oculars is sharply defined.
 - 10.3.2.3** Replace the slide with a business card or a piece of lens paper.
 - 10.3.2.4** Close the field diaphragm (iris diaphragm in the microscope base) so only a small point of light is visible on the card. This dot of light indicates the location of the center of the field of view.
 - 10.3.2.5** Mount the mercury lamp house on the microscope without the UV diffuser lens in place and turn on the mercury bulb.
 - 10.3.2.6** Remove the objective in the light path from the nosepiece. A primary (brighter) and secondary image (dimmer) of the mercury bulb arc should appear on the card after focusing the image with the appropriate adjustment.
 - 10.3.2.7** Using the lamp house adjustments, adjust the primary and secondary mercury bulb images so they are side by side (parallel to each other) with the transmitted light dot in between them.
 - 10.3.2.8** Reattach the objective to the nosepiece.
 - 10.3.2.9** Insert the diffuser lens into the light path between the mercury lamp house and the microscope.
 - 10.3.2.10** Turn off the transmitted light and replace the card with a slide of fluorescent material. Check the field for even fluorescent illumination.

Adjustment of the diffuser lens probably will be required. Additional slight adjustments as in Section 10.3.2.7 above may be required.

- 10.3.2.11** Maintain a log of the number of hours the UV bulb has been used. Never use the bulb for longer than it has been rated. Fifty-watt bulbs should not be used longer than 100 hours; 100-watt bulbs should not be used longer than 200 hours.

- 10.3.3** Transmitted bulb adjustment: The purpose of this procedure is to center the filament and ensure even field illumination. This procedure must be followed when the bulb is changed.

- 10.3.3.1** Remove the diffuser lens between the lamp and microscope or swing it out of the transmitted light path.

- 10.3.3.2** Using a prepared microscope slide and a 40X (or similar) objective, adjust the focus so the image in the oculars is sharply defined.

- 10.3.3.3** Without the ocular or Bertrand optics in place, view the pupil and filament image at the bottom of the tube.

- 10.3.3.4** Focus the lamp filament image with the appropriate adjustment on the lamp house.

- 10.3.3.5** Similarly, center the lamp filament image within the pupil with the appropriate adjustment(s) on the lamp house.

- 10.3.3.6** Insert the diffuser lens into the light path between the transmitted lamp house and the microscope.

- 10.3.4** Adjustment of the interpupillary distance and oculars for each eye: These adjustments are necessary so that eye strain is reduced to a minimum, and must be made for each individual using the microscope. Section 10.3.4.2 assumes use of a microscope with both oculars adjustable; Section 10.3.4.3 assumes use of a microscope with a single adjustable ocular. The procedure must be followed each time an analyst uses the microscope.

- 10.3.4.1** Interpupillary distance

- 10.3.4.1.1** Place a prepared slide on the microscope stage, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment knobs.

- 10.3.4.1.2** Using both hands, move the oculars closer together or farther apart until a single circle of light is observed while looking through the oculars with both eyes. Note interpupillary distance.

- 10.3.4.2** Ocular adjustment for microscopes capable of viewing a photographic frame through the viewing binoculars: This procedure assumes both oculars are adjustable.

- 10.3.4.2.1** Place a card between the right ocular and eye keeping both eyes open. Adjust the correction (focusing) collar on the left ocular by focusing the left ocular until it reads the same as the interpupillary distance. Bring an image located in the center of the field of view into as sharp a focus as possible.

- 10.3.4.2.2** Transfer the card to between the left eye and ocular. Again keeping both eyes open, bring the same image into as sharp a focus for the right eye as possible by adjusting the ocular correction (focusing) collar at the top of the right ocular.

10.3.4.3 Ocular adjustment for microscopes without binocular capability: This procedure assumes a single focusing ocular. The following procedure assumes that only the right ocular is capable of adjustment.

10.3.4.3.1 Place a card between the right ocular and eye keeping both eyes open. Using the fine adjustment, focus the image for the left eye to its sharpest point.

10.3.4.3.2 Transfer the card to between the left eye and ocular. Keeping both eyes open, bring the image for the right eye into sharp focus by adjusting the ocular collar at the top of the ocular without touching the coarse or fine adjustment.

10.3.5 Calibration of an ocular micrometer: This section assumes that a reticle has been installed in one of the oculars by a microscopy specialist and that a stage micrometer is available for calibrating the ocular micrometer (reticle). Once installed, the ocular reticle should be left in place. The more an ocular is manipulated the greater the probability is for it to become contaminated with dust particles. This calibration should be done for each objective in use on the microscope. If there is a top lens on the microscope, the calibration procedure must be done for the respective objective at each top lens setting. The procedure must be followed when the microscope is first used and each time the objective is changed.

10.3.5.1 Place the stage micrometer on the microscope stage, turn on the transmitted light, and focus the micrometer image using the coarse and fine adjustment knobs for the objective to be calibrated. Continue adjusting the focus on the stage micrometer so you can distinguish between the large (0.1 mm) and the small (0.01 mm) divisions.

10.3.5.2 Adjust the stage and ocular with the micrometer so the "0" line on the ocular micrometer is exactly superimposed on the "0" line on the stage micrometer.

10.3.5.3 Without changing the stage adjustment, find a point as distant as possible from the two 0 lines where two other lines are exactly superimposed.

10.3.5.4 Determine the number of ocular micrometer spaces as well as the number of millimeters on the stage micrometer between the two points of superimposition. For example: Suppose 48 ocular micrometer spaces equal 0.6 mm.

10.3.5.5 Calculate the number of mm/ocular micrometer space. For example:

$$\frac{0.6 \text{ mm}}{48 \text{ ocular micrometer spaces}} = \frac{0.0125 \text{ mm}}{\text{ocular micrometer space}}$$

10.3.5.6 Because most measurements of microorganisms are given in μm rather than mm, the value calculated above must be converted to μm by multiplying it by 1000 $\mu\text{m}/\text{mm}$. For example:

$$\frac{0.0125 \text{ mm}}{\text{ocular micrometer space}} \times \frac{1,000 \mu\text{m}}{\text{mm}} = \frac{12.5 \mu\text{m}}{\text{ocular micrometer space}}$$

- 10.3.5.7** Follow the procedure below for each objective. Record the information as shown in the example below and keep the information available at the microscope.

Item no.	Objective power	Description	No. of ocular micrometer spaces	No. of stage micrometer mm ¹	µm/ocular micrometer space ²
1	10X	N.A. ³ =			
2	20X	N.A.=			
3	40X	N.A.=			
4	100X	N.A.=			

¹1000 µm/mm

²(Stage micrometer length in mm × (1000 µm/mm)) ÷ no. ocular micrometer spaces

³N.A. refers to numerical aperture. The numerical aperture value is engraved on the barrel of the objective.

- 10.3.6** Köhler illumination: This section assumes that Köhler illumination will be established for only the 100X oil DIC objective that will be used to identify internal morphological characteristics in *Cryptosporidium* oocysts and *Giardia* cysts. If more than one objective is to be used for DIC, then each time the objective is changed, Köhler illumination must be reestablished for the new objective lens. Previous sections have adjusted oculars and light sources. This section aligns and focuses the light going through the condenser underneath the stage at the specimen to be observed. If Köhler illumination is not properly established, then DIC will not work to its maximal potential. These steps need to become second nature and must be practiced regularly until they are a matter of reflex rather than a chore. The procedure must be followed each time an analyst uses the microscope and each time the objective is changed.

- 10.3.6.1** Place a prepared slide on the microscope stage, place oil on the slide, move the 100X oil objective into place, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment knobs.
- 10.3.6.2** At this point both the radiant field diaphragm in the microscope base and the aperture diaphragm in the condenser should be wide open. Now close down the radiant field diaphragm in the microscope base until the lighted field is reduced to a small opening.
- 10.3.6.3** Using the condenser centering screws on the front right and left of the condenser, move the small lighted portion of the field to the center of the visual field.
- 10.3.6.4** Now look to see whether the leaves of the iris field diaphragm are sharply defined (focused) or not. If they are not sharply defined, then they can be focused distinctly by changing the height of the condenser up and down with the condenser focusing knob while you are looking through the binoculars. Once you have accomplished the precise focusing of the radiant field diaphragm leaves, open the radiant field diaphragm until the leaves just disappear from view.
- 10.3.6.5** The aperture diaphragm of the condenser ~~should is~~ now ~~be~~ adjusted to make it compatible with the total numerical aperture of the optical system. This is done by removing an ocular, looking into the tube at the rear focal plane of the objective, and stopping down the aperture diaphragm iris leaves until they are visible just inside the rear plane of the objective.

- 10.3.6.6** After completing the adjustment of the aperture diaphragm in the condenser, return the ocular to its tube and proceed with the adjustments required to establish DIC.

10.4 Microscope cleaning procedure

- 10.4.1** Use canned air to remove dust from the lenses, filters, and microscope body.
- 10.4.2** Use a Kimwipe-dampened with a microscope cleaning solution (MCS) (consisting of 2 parts 90% isopropanol and 1 part acetone) to wipe down all surfaces of the microscope body. Dry off with a clean, dry Kimwipe.
- 10.4.3** Protocol for cleaning oculars and condenser
- 10.4.3.1** Use a new, clean Q-tip dampened with MCS to clean each lense. Start at the center of the lens and spiral the Q-tip outward using little to no pressure. Rotate the Q-tip head while spiraling to ensure a clean surface is always contacting the lens.
- 10.4.3.2** Repeat the procedure using a new, dry Q-tip.
- 10.4.3.3** Repeat Sections 10.4.3.1 and 10.4.3.2.
- 10.4.3.4** Remove the ocular and repeat the cleaning procedure on the bottom lens of the ocular.
- 10.4.4** Protocol for cleaning objective lenses
- 10.4.4.1** Wipe 100X oil objective with lense paper to remove the bulk of the oil from the objective.
- 10.4.4.2** Hold a new Q-tip dampened with MCS at a 45° angle on the objective and twirl.
- 10.4.4.3** Repeat Sections 10.4.4.2 with a new, dry Q-tip.
- 10.4.4.4** Repeat Sections 10.4.4.2 and 10.4.4.3.
- 10.4.4.5** Clean all objectives whether they are used or not.
- 10.4.5** Protocol for cleaning light source lens and filters
- 10.4.5.1** Using a Kimwipe dampened with microscope cleaning solution, wipe off the surface of each lens and filter.
- 10.4.5.2** Repeat the procedure using a dry Kimwipe.
- 10.4.5.3** Repeat Sections 10.4.5.1 and 10.4.5.2.
- 10.4.6** Protocol for cleaning microscope stage
- 10.4.6.1** Using a Kimwipe dampened with microscope cleaning solution, wipe off the stage and stage clip. Be sure to clean off any residual immersion oil or fingernail polish. Remove the stage clip if necessary to ensure that it is thoroughly cleaned.
- 10.4.7** Use 409 and a paper towel to clean the bench top surrounding the microscope.
- 10.4.8** Frequency
- 10.4.8.1** Perform Sections 10.4.2, 10.4.3, 10.4.4, 10.4.5 and 10.4.7 after each microscope session.
- 10.4.8.2** Perform complete cleaning each week.
- 10.5** Protozoa libraries: Each laboratory is encouraged to develop libraries of photographs and drawings for identification of protozoa.
- 10.5.1** Take color photographs of *Cryptosporidium* oocysts and *Giardia* cysts by FA, and 4',6-diamidino-2-phenylindole (DAPI), and DIC that the analysts (Section 22.2) determine are accurate (Section 15.2).

- 10.5.2** Similarly, take color photographs of interfering organisms and materials by FA, ~~and~~ DAPI, ~~and~~ DIC that the analysts believe are not *Cryptosporidium* oocysts or *Giardia* cysts. Quantify the size, shape, microscope settings, and other characteristics that can be used to differentiate oocysts and cysts from interfering debris and that will result in ~~accurate positive identification of DAPI-positive or negative organisms.~~
- 10.6** Verification of ~~analyst~~ performance: Until standard reference materials, such as National Institute of Standards and Technology standard reference materials, are available that contain a reliable number of DAPI positive or negative oocysts and cysts, this method shall rely upon the ability of the analyst for identification and enumeration of oocysts and cysts.
- 10.6.1** At least monthly when microscopic examinations are being performed, the laboratory shall prepare a slide containing 40 to 100 oocysts and 40 to 100 cysts. More than 50% of the oocysts and cysts must be DAPI positive ~~and undamaged under DIC.~~
- 10.6.2** Each analyst shall determine the total number of oocysts and cysts and the number that are DAPI positive and DAPI negative ~~for the entire slide. For 10 oocysts and 10 cysts, each analyst shall determine using the slide prepared in Section 10.5.1: the number of nuclei by DAPI and the DIC category (empty, containing amorphous structures, or containing identifiable internal structures) of each.~~
- 10.6.3** Requirements for laboratories with multiple analysts
- 10.6.3.1** The total number and the number of DAPI positive ~~or~~ ~~and~~ DAPI negative oocysts and cysts determined by each analyst (Section 10.5.2.) must be within $\pm 10\%$ of each other. If the number is not within this range, the analysts must identify the source of any variability between analysts' examination criteria, prepare a new slide, and repeat the performance verification (Sections 10.5.1 to 10.5.2). ~~Differences in the number of nuclei by DAPI and in DIC categorizations among analysts must be discussed and resolved, and these resolutions must be documented.~~
- 10.6.3.2** Document the date, name(s) of analyst(s), number of total, DAPI positive or negative oocysts and cysts determined by the analyst(s), whether the test was passed/failed and the results of attempts before the test was passed.
- 10.6.3.3** Only after an analyst has passed the criteria in Section 10.6.3, may oocysts and cysts in QC samples and field samples be identified and enumerated.
- 10.6.4** ~~Laboratories with only one analyst should maintain a protozoa library (Section 10.5) and compare the results of the examinations performed in Sections 10.6.1 and 10.6.2 to photographs of oocysts and cysts and interfering organisms to verify that examination results are consistent with these references. These laboratories also should perform repetitive counts of a single verification slide for FITC and DAPI. These laboratories should also coordinate with other laboratories to share slides and compare counts.~~

11.0 Oocyst and Cyst Suspension Enumeration and Spiking

- 11.1** This method requires routine analysis of spiked QC samples to demonstrate acceptable initial and ongoing laboratory and method performance (initial precision and recovery samples [Section 9.4], matrix spike and matrix spike duplicate samples [Section 9.5], and ongoing precision and recovery samples [Section 9.7]). The organisms used for these samples must be enumerated to calculate recoveries and precision. EPA recommends that flow cytometry be used for this enumeration, rather than manual techniques. Flow cytometer-sorted spikes generally are characterized by a relative standard deviation of $\leq 2.5\%$, versus greater variability for manual enumeration techniques (Reference 20.8). Guidance on preparing spiking suspensions using a flow cytometer is provided in Section 11.2. Manual enumeration procedures are provided in

Section 11.3. The procedure for spiking bulk samples in the laboratory is provided in Section 11.4.

11.2 Flow cytometry enumeration guidelines. Although it is unlikely that many laboratories performing Method 1623 will have direct access to a flow cytometer for preparing spiking suspensions, flow-sorted suspensions are available from commercial vendors and other sources (Section 7.10.1). The information provided in Sections 11.2.1 through 11.2.4 is simply meant as a guideline for preparing spiking suspensions using a flow cytometer. Laboratories performing flow cytometry must develop and implement detailed standardized protocols for calibration and operation of the flow cytometer.

11.2.1 Spiking suspensions should be prepared using unstained organisms that have not been heat-fixed or formalin-fixed.

11.2.2 Spiking suspensions should be prepared using *Cryptosporidium parvum* oocysts <3 months old, and *Giardia intestinalis* cysts <2 weeks old.

11.2.3 Initial calibration. Immediately before sorting spiking suspensions, an initial calibration of the flow cytometer should be performed by conducting 10 sequential sorts directly onto membranes or well slides. The oocyst and cyst levels used for the initial calibration should be the same as the levels used for the spiking suspensions. Each initial calibration sample should be stained and manually counted microscopically and the manual counts used to verify the accuracy of the system. The relative standard deviation (RSD) of the 10 counts should be $\leq 2.5\%$. If the RSD is $> 2.5\%$, the laboratory should perform the initial calibration again, until the RSD of the 10 counts is $\leq 2.5\%$. In addition to counting the organisms, the laboratory also should evaluate the quality of the organisms using DAPI and DIC to confirm that the organisms are in good condition.

11.2.4 Ongoing calibration. When sorting the spiking suspensions for use in QC samples, the laboratory should perform ongoing calibration samples at a 10% frequency, at a minimum. The laboratory should sort the first run and every eleventh sample directly onto a membrane or well slide. Each ongoing calibration sample should be stained and manually counted microscopically and the manual counts used to verify the accuracy of the system. The mean of the ongoing calibration counts also should be used as the estimated spike dose, if the relative standard deviation (RSD) of the ongoing calibration counts is $\leq 2.5\%$. If the RSD is $> 2.5\%$, the laboratory should discard the batch.

11.2.5 Method blanks. Depending on the operation of the flow cytometer, method blanks should be prepared and examined at the same frequency as the ongoing calibration samples (Section 11.2.4).

11.2.6 Holding time criteria. Flow-cytometer-sorted spiking suspensions (Sections 7.10.1 and 11.2) used for spiked quality control (QC) samples (Section 9) must be used within the expiration date noted on the suspension. ~~The holding time specified by the flow cytometry laboratory should be determined based on a holding time study. Laboratories should use flow-cytometer-sorted spiking suspensions containing live organisms within two weeks of preparation at the flow cytometry laboratory.~~

11.3 Manual enumeration procedures. Two sets of manual enumerations are required per organism before purified *Cryptosporidium* oocyst and *Giardia* cyst stock suspensions (~~Sections 7.9.2.1 and 7.9.2.2~~ **Section 7.10.2**) received from suppliers can be used to spike samples in the laboratory. First, the stock suspension must be diluted and enumerated (Section 11.3.3) to yield a suspension at the appropriate oocyst or cyst concentration for spiking (spiking suspension). Then, 10 aliquots of spiking suspension must be enumerated to calculate a mean spike dose. Spiking suspensions can be enumerated using hemacytometer chamber counting (Section 11.3.4), well slide counting (Section 11.3.5), or membrane filter counting (Section 11.3.6).

11.3.1 Precision criteria. The relative standard deviation (RSD) of the calculated mean spike dose for manually enumerated spiking suspensions must be $\leq 16\%$ for *Cryptosporidium* and $\leq 19\%$ for *Giardia* before proceeding (these criteria are based on the pooled RSDs of

105 manual *Cryptosporidium* enumerations and 104 manual *Giardia* enumerations submitted by 20 different laboratories under the EPA Protozoa Performance Evaluation Program).

11.3.2 Holding time criteria. Manually enumerated spiking suspensions must be used within 24 hours of enumeration of the spiking suspension if the hemacytometer chamber technique is used (Section 11.3.4); or within 24 hours of application of the spiking suspension or membrane filter to the slides if the well slide or membrane filter enumeration technique is used (Sections 11.3.5 and 11.3.6).

11.3.3 Enumerating and diluting stock suspensions

11.3.3.1 Purified, concentrated stock suspensions (Sections 7.10.2.1 and 7.10.2.2) must be diluted and enumerated before the diluted suspensions are used to spike samples in the laboratory. Stock suspensions should be diluted with reagent water/Tween-20, 0.01% (Section 7.10.2.3), to a concentration of 20 to 50 organisms per large hemacytometer square before proceeding to Section 11.3.3.2.

11.3.3.2 Apply a clean hemacytometer coverslip (Section 6.4.5) to the hemacytometer and load the hemacytometer chamber with 10 µL of vortexed suspension per chamber. If this operation has been properly executed, the liquid should amply fill the entire chamber without bubbles or overflowing into the surrounding moats. Repeat this step with a clean, dry hemacytometer and coverslip if loading has been incorrectly performed. See Section 11.3.3.13, below, for the hemacytometer cleaning procedure.

11.3.3.3 Place the hemacytometer on the microscope stage and allow the oocysts or cysts to settle for 2 minutes. Do not attempt to adjust the coverslip, apply clips, or in any way disturb the chamber after it has been filled.

11.3.3.4 Use 200X magnification.

11.3.3.5 Move the chamber so the ruled area is centered underneath ~~it~~ the objective.

11.3.3.6 Move the objective close to the coverslip while watching it from the side of the microscope, rather than through the microscope.

11.3.3.7 Focus up from the coverslip until the hemacytometer ruling appears.

11.3.3.8 At each of the four corners of the chamber is a 1-square-mm area divided into 16 squares in which organisms are to be counted (Figure 1). Beginning with the top row of four squares, count with a hand-tally counter in the directions indicated in Figure 2. Avoid counting organisms twice by counting only those touching the top and left boundary lines. Count each square millimeter in this fashion.

11.3.3.9 Use the following formula to determine the number of organisms per mL of suspension:

$$\frac{\text{number of organisms counted}}{\text{number of mm}^2 \text{ counted}} \times \frac{10}{1 \text{ mm}} \times \frac{\text{dilution factor}}{1} \times \frac{1000 \text{ mm}^2}{1 \text{ }\mu\text{L mL}} = \frac{\text{number of organisms}}{\text{ }\mu\text{L mL}}$$

11.3.3.10 Record the result on a hemacytometer data sheet.

- 11.3.3.11** A total of six different hemacytometer chambers must be loaded, counted, and averaged for each suspension to achieve optimal counting accuracy.
- 11.3.3.12** Based on the hemacytometer counts, the stock suspension should be diluted to a final concentration of between ~~8000 and 12,000 organisms per mL~~ (80 to 120 organisms per ~~10~~ μ L); however, ranges as great as ~~5000 to 15,000 organisms per mL~~ (50 to 150 organisms per ~~10~~ μ L) can be used.

NOTE: If the diluted stock suspensions (the spiking suspensions) will be enumerated using hemacytometer chamber counts (Section 11.3.4) or membrane filter counts (Section 11.3.6), then the stock suspensions should be diluted with 0.01% Tween-20. If the spiking suspensions will be enumerated using well slide counts (Section ~~11.3.3~~ 11.3.5), then the stock suspensions should be diluted in reagent water.

To calculate the volume (in μ L) of stock suspension required per mL of reagent water (or reagent water/Tween-20, 0.01%), use the following formula:

$$\text{volume of stock suspension } (\mu\text{L}) \text{ required} = \frac{\text{required number of organisms} \times \cancel{1000 \mu\text{L}}}{\text{number of organisms} / \cancel{\mu\text{mL}} \text{ of stock suspension}}$$

If the volume is less than 10 μ L, an additional dilution of the stock suspension is recommended before proceeding.

To calculate the dilution factor needed to achieve the required number of organisms per 10 μ L, use the following formula:

$$\text{total volume } (\mu\text{L}) = \frac{\text{number of organisms required} \times 10\mu\text{L}}{\text{predicted number of organisms per } 10\mu\text{L} \text{ (80 to 120)}}$$

To calculate the volume of reagent water (or reagent water/Tween-20, 0.01%) needed, use the following formula:

$$\text{reagent water volume } (\mu\text{L}) = \text{total volume } (\mu\text{L}) - \text{stock suspension volume required } (\mu\text{L})$$

- 11.3.3.13** After each use, the hemacytometer and coverslip must be cleaned immediately to prevent the organisms and debris from drying on it. Since this apparatus is precisely machined, abrasives cannot be used to clean it, as they will disturb the flooding and volume relationships.
- 11.3.3.13.1** Rinse the hemacytometer and cover glass first with tap water, then 70% ethanol, and finally with acetone.
- 11.3.3.13.2** Dry and polish the hemacytometer chamber and cover glass with lens paper. Store it in a secure place.

11.3.3.14 Several factors are known to introduce errors into hemacytometer counts, including:

- Inadequate mixing of suspension before flooding the chamber
- Irregular filling of the chamber, trapped air bubbles, dust, or oil on the chamber or coverslip
- Total number of organisms counted is too low to provide statistical confidence in the result
- Error in recording tally
- Calculation error; failure to consider dilution factor, or area counted
- Inadequate cleaning and removal of organisms from the previous count
- Allowing filled chamber to sit too long, so that the chamber suspension dries and concentrates.

11.3.4 Enumerating spiking suspensions using a hemacytometer chamber

NOTE: Spiking suspensions enumerated using a hemacytometer chamber must be used within 24 hours of enumeration.

11.3.4.1 Vortex the tube containing the spiking suspension (diluted stock suspension; Section 11.3.3) for a minimum of 2 minutes. Gently invert the tube three times.

11.3.4.2 To an appropriate-size beaker containing a stir bar, add enough spiking suspension to perform all spike testing and the enumeration as described. The liquid volume and beaker relationship should be such that a spinning stir bar does not splash the sides of the beaker, the stir bar has unimpeded rotation, and there is enough room to draw sample from the beaker with a 10- μ L micropipette without touching the stir bar. Cover the beaker with a watch glass or petri dish to prevent evaporation between sample withdrawals.

11.3.4.3 Allow the beaker contents to stir for a minimum of 30 minutes before beginning enumeration.

11.3.4.4 While the stir bar is still spinning, remove a 10- μ L aliquot and carefully load one side of the hemacytometer. Count all organisms on the platform, at 200X magnification using phase-contrast or darkfield microscopy. The count must include the entire area under the hemacytometer, not just the four outer 1-mm² squares. Repeat this procedure nine times. This step allows confirmation of the number of organisms per 10 μ L (Section 11.3.3.12). Based on the 10 counts, calculate the mean, standard deviation, and RSD of the counts. Record the counts and the calculations on a spiking suspension enumeration form. The relative standard deviation (RSD) of the calculated mean spike dose must be $\leq 16\%$ for *Cryptosporidium* and $\leq 19\%$ for *Giardia* before proceeding. If the RSD is unacceptable, or the mean number is outside the expected range, add additional oocysts from stock suspension or dilute the contents of the beaker appropriately with reagent water. Repeat the process to confirm counts. Refer to Section 11.3.3.14 for factors that may introduce errors.

11.3.5 Enumerating spiking suspensions using well slides

NOTE: Spiking suspensions enumerated using well slides must be used within 24 hours of application of the spiking suspension to the slides.

- 11.3.5.1** Prepare well slides for sample screening and label the slides. ~~Remove well slides from cold storage and lay the slides on a flat surface for 15 minutes to allow them to warm to room temperature.~~
- 11.3.5.2** Vortex the tube containing the spiking suspension (diluted stock suspension; Section 11.3.3) for a minimum of 2 minutes. Gently invert the tube three times.
- 11.3.5.3** Remove a 10- μ L aliquot from the spiking suspension and apply it to the center of a well.
- 11.3.5.4** Before removing subsequent aliquots, cap the tube and gently invert it three times to ensure that the oocysts or cysts are in suspension.
- 11.3.5.5** Ten wells must be prepared and counted, and the counts averaged, to sufficiently enumerate the spike dose. Air-dry the well slides. Because temperature and humidity varies from laboratory to laboratory, no minimum time is specified. However, the laboratory must take care to ensure that the sample has dried completely before staining to prevent losses during the rinse steps. A slide warmer set at 35°C to 42°C also can be used.
- 11.3.5.6** Positive and negative controls must be prepared.
- 11.3.5.6.1** For the positive control, pipette 10 μ L of positive antigen or 200 to 400 intact oocysts or cysts to the center of a well and distribute evenly over the well area.
- 11.3.5.6.2** For the negative control, pipette 50 μ L of PBS onto the center of a well and spread it over the well area with a pipette tip.
- 11.3.5.6.3** Air-dry the control slides.
- ~~**11.3.5.7** Apply 50- μ L of absolute methanol to each well containing the dried sample and allow to air-dry for 3 to 5 minutes.~~
- 11.3.5.7** Follow the manufacturer's instructions (Section 7.6) in applying the stain to the slide.
- 11.3.5.8** Place the slides in a humid chamber in the dark and incubate ~~according to manufacturer's directions. at room temperature for approximately 30 minutes.~~ The humid chamber consists of a tightly sealed plastic container containing damp paper towels on top of which the slides are placed.
- 11.3.5.9** Apply one drop of wash buffer (prepared according to the manufacturer's instructions [Section 7.6]) to each well. Tilt each slide on a clean paper towel, long edge down. Gently aspirate the excess detection reagent from below the well using a clean Pasteur pipette or absorb with a paper towel or other absorbent material. Avoid disturbing the sample.

NOTE: If using the Merifluor stain (Section 7.6.1), do not allow slides to dry completely.

- 11.3.5.10** Add mounting medium (Section 7.8) to each well.
- 11.3.5.11** Apply a cover slip. Use a tissue to remove excess mounting fluid from the edges of the coverslip. Seal the edges of the coverslip onto the slide using clear nail polish.

- 11.3.5.12** Record the date and time that staining was completed. If slides will not be read immediately, store in a humid chamber in the dark at 0°C to 8°C < 10°C and not frozen until ready for examination.
- 11.3.5.13** After examination of the 10 wells, calculate the mean, standard deviation, and RSD of the 10 replicates. Record the counts and the calculations on a spiking suspension enumeration form. The relative standard deviation (RSD) of the calculated mean spike dose must be ≤16% for *Cryptosporidium* and ≤19% for *Giardia* before proceeding. If the RSD is unacceptable, or the mean number is outside the expected range, add additional oocysts from stock suspension or dilute the contents of the beaker appropriately with reagent water. Repeat the process to confirm counts.

11.3.6 Enumeration of spiking suspensions using membrane filters

NOTE: Spiking suspensions enumerated using membrane filters must be used within 24 hours of application of the filters to the slides.

- 11.3.6.1** Precoat the glass funnels with Sigmacote® by placing the funnel in a large petri dish and applying 5-mL of Sigmacoat® to the funnel opening using a pipette and allowing it to run down the inside of the funnel. Repeat for all funnels to be used. The pooled Sigmacoat® may be returned to the bottle for re-use. Place the funnels at 35°C or 41°C for approximately 5 minutes to dry.
- 11.3.6.2** Place foil around the bottoms of the 100 × 15 mm petri dishes.
- 11.3.6.3** Filter-sterilize (Section 6.19) approximately 10 mL of PBS pH 7.2 (Section 7.9.4 7.6.4). Dilute detection reagent (Section 7.7) as per manufacturer's instructions using sterile PBS. Multiply the anticipated number of filters to be stained by 100 mL to calculate total volume of stain required. Divide the total volume required by 5 to obtain the microliters of antibody necessary. Subtract the volume of antibody from the total stain volume to obtain the required microliters of sterile PBS to add to the antibody.
- 11.3.6.4** Label the tops of foil-covered, 60 × 15 mm petri dishes for 10 spiking suspensions plus positive and negative staining controls and multiple filter blanks controls (one negative control, plus a blank after every five sample filters to control for carry-over). Create a humid chamber by laying damp paper towels on the bottom of a stain tray (the inverted foil-lined petri dishes will protect filters from light and prevent evaporation during incubation).
- 11.3.6.5** Place a decontaminated and cleaned filter holder base (Section 6.4.8.1) into each of the three ports of the vacuum manifold (Section 6.4.8.2).
- 11.3.6.6** Pour approximately 10 mL of 0.01% Tween 20 into a 60 × 15 mm petri dish.
- 11.3.6.7** Using forceps, moisten a 1.2-µm cellulose-acetate support membrane (Section 6.4.8.3) in the 0.01% Tween 20 and place it on the fritted glass support of one of the filter bases. Moisten a polycarbonate filter (Section 6.4.8.4) the same way and position it on top of the cellulose-acetate support membrane. Carefully clamp the glass funnel to the loaded filter support. Repeat for the other two filters.
- 11.3.6.8** Add 5 mL of 0.01% Tween 20 to each of the three filtration units and allow to stand.

- 11.3.6.9** Vortex the tube containing the spiking suspension (diluted stock suspension; Section 11.3.3) for a minimum of 2 minutes. Gently invert the tube three times.
- 11.3.6.10** Using a micropipettor, sequentially remove two, 10- μ L aliquots from the spiking suspension and pipet into the 5 mL of 0.01% Tween 20 standing in the unit. Rinse the pipet tip twice after each addition. Apply 10 μ L of 0.01% Tween 20 to the third unit to serve as the negative control. Apply vacuum at 2" Hg and allow liquid to drain to miniscus, then close off vacuum. Pipet 10 mL of reagent water into each funnel and drain to miniscus, closing off the vacuum. Repeat the rinse and drain all fluid, close off the vacuum.
- 11.3.6.11** Pipet 100 mL of diluted antibody to the center of the bottom of a 60 \times 15 mm petri dish for each sample.
- 11.3.6.12** Unclamp the top funnel and transfer each cellulose acetate support membrane/ polycarbonate filter combination onto the drop of stain using forceps (apply each membrane/filter combination to a different petri dish containing stain). Roll the filter into the drop to exclude air. Place the small petri dish containing the filter onto the damp towel and cover with the corresponding labeled foil-covered top. Incubate for approximately 45 minutes at room temperature.
- 11.3.6.13** Reclamp the top funnels, apply vacuum and rinse each three times, each time with 20 mL of reagent water.
- 11.3.6.14** Repeat Sections 11.3.6.4 through 11.3.6.10 for the next three samples (if that the diluted spiking suspension has sat less than 15 minutes, reduce the suspension vortex time to 60 seconds). Ten, 10- μ L spiking suspension aliquots must be prepared and counted, and the counts averaged, to sufficiently enumerate the spike dose. Include a filter blank sample at a frequency of every five samples; rotate the position of filter blank to eventually include all three filter placements.
- 11.3.6.15** Repeat Sections 11.3.6.4 through 11.3.6.10 until the 10- μ L spiking suspensions have been filtered. The last batch should include a 10- μ L 0.01 Tween 20 blank control and 20 μ L of positive control antigen as a positive staining control.
- 11.3.6.16** Label slides. After incubation is complete, for each sample, transfer the cellulose acetate filter support and polycarbonate filter from drop of stain and place on fritted glass support. Cycle vacuum on and off briefly to remove excess fluid. Peel the top polycarbonate filter off the supporting filter and place on labeled slide. Discard cellulose acetate filter support. Mount and apply coverslips to the filters immediately to avoid drying.
- 11.3.6.17** To each slide, add 20 μ L of mounting medium (Section 7.8).
- 11.3.6.18** Apply a coverslip. Seal the edges of the coverslip onto the slide using clear nail polish. (Sealing may be delayed until cover slips are applied to all slides.)
- 11.3.6.19** Record the date and time that staining was completed. If slides will not be read immediately, store sealed slides in a closed container in the dark at ~~0°C to~~ $< 8^{\circ}\text{C}$ (but not frozen) until ready for examination.
- 11.3.6.20** After examination of the 10 slides, calculate the mean, standard deviation, and RSD of the 10 replicates. Record the counts and the calculations on a spiking suspension enumeration form. The relative standard deviation (RSD) of the calculated mean spike dose must be

≤16% for *Cryptosporidium* and ≤19% for *Giardia* before proceeding. If the RSD is unacceptable, or the mean number is outside the expected range, add additional oocysts from stock suspension or dilute the contents of the beaker appropriately with reagent water. Repeat the process to confirm counts.

11.3.6.21 If oocysts or cysts are detected on the filter blanks, modify the rinse procedure to ensure that no carryover occurs and repeat enumeration.

11.4 Procedure for spiking samples in the laboratory with enumerated spiking suspensions.

11.4.1 Arrange a bottom-dispensing container to feed the filter ~~or insert the influent end of the tube connected to the filter through the top of a carboy to allow siphoning of the sample.~~

11.4.2 For initial precision and recovery (Section 9.4) and ongoing precision and recovery (Section 9.7) samples, fill the container with ~~10 L of reagent water or~~ a volume of reagent water equal to the volume of the field samples analyzed in the analytical batch. For matrix spike samples (Section 9.5), fill the container with the field sample to be spiked. Continuously mix the sample (using a stir bar and stir plate for smaller-volume samples and alternate means for larger-volume samples).

11.4.3 ~~Follow the procedures in Section 11.4.3.1 for flow cytometer–enumerated suspensions and the procedures in Section 11.4.3.2 for manually enumerated suspensions. Vortex the spiking suspension(s) (Section 11.2 or Section 11.3) for a minimum of 2 minutes.~~

11.4.3.1 For flow cytometer–enumerated suspensions (where the entire volume of a spiking suspension tube will be used):

11.4.3.1.1 Add 400 µL of Antifoam A to 100 mL of reagent water, and mix well to emulsify.

11.4.3.1.2 Add 500 µL of the diluted antifoam to the tube containing the spiking suspension and vortex for ~~30 seconds~~ **2 minutes**.

11.4.3.1.3 Pour the suspension into the sample container.

11.4.3.1.4 Add 20 mL of reagent water to the empty tube, cap, vortex 10 seconds to rinse, and add the rinsate to the carboy.

11.4.3.1.5 Repeat this rinse using another 20 mL of reagent water.

11.4.3.1.6 Record the estimated number of organisms spiked, the date and time the sample was spiked, and the sample volume spiked on a bench sheet. Proceed to Section 11.4.4.

11.4.3.2 For manually enumerated spiking suspensions:

11.4.3.2.1 ~~Vortex the spiking suspension(s) (Section 11.2 or Section 11.3) for a minimum of 30 seconds.~~

11.4.3.2.2 Rinse a pipette tip with 0.01% Tween-20 once, then rinse with the well-mixed spiking suspension a minimum of five times before pulling an aliquot to be used to spike the container.

11.4.3.2.3 Add the spiking suspension(s) to the carboy, delivering the aliquot below the surface of the water.

11.4.3.2.4 Record the estimated number of organisms spiked, the date and time the sample was spiked, and the sample volume spiked on a bench sheet. Proceed to Section 11.4.4

- 11.4.4** Allow the spiking suspensions to mix for approximately 1 minute in the container.
- 11.4.5** Turn on the pump and allow the flow rate to stabilize. Set flow at the rate designated for the filter being used. As the carboy is depleted, check the flow rate and adjust if necessary.
- 11.4.6** When the water level approaches the discharge port of the carboy, tilt the container so that it is completely emptied. At that time, turn off the pump and add sufficient reagent water to the container to rinse. Swirl the contents to rinse down the sides. **Additional rinses may be performed.**
- 11.4.7** Turn on the pump. Allow all of the water to flow through the filter and turn off the pump.

12.0 Sample Filtration and Elution

- 12.1** A water sample is filtered according to the procedures in Section 12.2 or Section 12.3. Alternate procedures may be used if the laboratory first demonstrates that the alternate procedure provides equivalent or superior performance per Section 9.1.2.

NOTE: Sample elution must be initiated within 96 hours of sample collection (if shipped to the laboratory as a bulk sample) or filtration (if filtered in the field).

- 12.2** Capsule filtration (adapted from Reference 20.9). This procedure was validated using 10-L sample volumes (for the original Envirochek™ filter) and 50-L sample volumes (for the Envirochek™ HV filter). Alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and source water samples (Section 9.1.2).

NOTE: The filtration procedures specified in Section 12.2.1 - 12.2.5.3 are specific to laboratory filtration of a bulk sample, and reflect the procedures used during the interlaboratory validation of this method (Reference 20.10). These procedures may require modification if samples will be filtered in the field.

12.2.1 Flow rate adjustment

- 12.2.1.1** Connect the sampling system, minus the capsule, to a carboy filled with reagent water (Figure 3).
- 12.2.1.2** Turn on the pump and adjust the flow rate to 2.0 L/min.
- 12.2.1.3** Allow 2 to 10 L of reagent water to flush the system. Adjust the pump speed as required during this period. Turn off the pump when the flow rate has been adjusted.

- 12.2.2** Install the capsule filter in the line, securing the inlet and outlet ends with the appropriate clamps/fittings.

- 12.2.3** Record the sample number, sample turbidity (if not provided with the field sample), sample type, and sample filtration start date and time on a bench sheet.

12.2.4 Filtration

- 12.2.4.1** **Mix the sample well by shaking and** connect the sampling system to the field carboy of sample water, or transfer the sample water to the laboratory carboy used in Section 12.2.1.1. If the sample will be filtered from a field carboy, a spigot (Section 6.2.1) can be used with the carboy to facilitate sample filtration.

NOTE: If the bulk field sample is transferred to a laboratory carboy, the laboratory carboy must be cleaned and disinfected before it is used with another field sample.

- 12.2.4.2** Place the drain end of the sampling system tubing into an empty graduated container with a capacity of 10 to 15 L, calibrated at 9.0, 9.5, 10.0, 10.5, and 11.0 L (Section 6.18). This container will be used to determine the sample volume filtered. Alternately, connect a flow meter (Section 6.3.4) downstream of the filter, and record the initial meter reading.
- 12.2.4.3** Allow the carboy discharge tube and capsule to fill with sample water **by gravity**. Vent residual air using the bleed valve/vent port, gently shaking or tapping the capsule, if necessary. Turn on the pump to start water flowing through the filter. Verify that the flow rate is 2 L/min.
- 12.2.4.4** After all of the sample has passed through the filter, turn off the pump. Allow the pressure to decrease until flow stops. (If the sample was filtered in the field, and excess sample remains in the filter **capsule** upon receipt in the laboratory, pull the remaining sample volume through the filter before eluting the filter [Section 12.2.6].)
- 12.2.5** Disassembly
- 12.2.5.1** Disconnect the inlet end of the capsule filter assembly while maintaining the level of the inlet fitting above the level of the outlet fitting to prevent backwashing and the loss of oocysts and cysts from the filter. Restart the pump and allow as much water to drain as possible. Turn off the pump.
- 12.2.5.2** Based on the water level in the graduated container **and ½-L hash marks** or meter reading, record the volume filtered on the bench sheet to the nearest quarter liter. Discard the contents of the graduated container.
- 12.2.5.3** Loosen the outlet fitting, then cap the inlet and outlet fittings.

12.2.6 Elution

***NOTE:** The laboratory must complete the elution, concentration, and purification (Sections 12.2.6 through 13.3.3.11) in one work day. It is critical that these steps be completed in one work day to minimize the time that any target organisms present in the sample sit in eluate or concentrated matrix. This process ends with the application of the purified sample on the slide for drying.*

- 12.2.6.1** Setup
- 12.2.6.1.1** Assemble the laboratory shaker with the clamps aligned vertically so that the filters will be aligned horizontally. Extend the clamp arms to their maximum distance from the horizontal shaker rods to maximize the shaking action.
- 12.2.6.1.2** Prepare sufficient elution buffer so that all samples to be eluted that day can be eluted with the same batch of buffer. Elution may require up to 275 mL of buffer per sample.
- 12.2.6.1.3** Designate at least one 250-mL conical centrifuge tube for each sample and label with the sample number.
- 12.2.6.2** Elution
- 12.2.6.2.1** Record the elution date and time on the bench sheet. Using a ring stand or other means, clamp each capsule in a vertical position with the inlet end up. **Remove the inlet cap and allow the liquid level to stabilize.**

- 12.2.6.2.2** Pour elution buffer through the inlet fitting. **Remove the inlet cap and allow the liquid level to stabilize.** Sufficient elution buffer must be added to cover the pleated white membrane with buffer solution. Replace the inlet cap **and clamp the cap in place.**
- 12.2.6.2.3** Securely clamp the capsule in one of the clamps on the laboratory shaker with the bleed valve positioned at the top on a vertical axis (in the 12 o'clock position). Turn on the shaker and set the speed to maximum (approximately 900 rpm). Agitate the capsule for approximately 5 minutes. Time the agitation using a lab timer, rather than the timer on the shaker to ensure accurate time measurement.
- 12.2.6.2.4** Remove the filter from the shaker, remove the inlet cap, and pour the contents of the capsule into the 250-mL conical centrifuge tube.
- 12.2.6.2.5** Clamp the capsule vertically with the inlet end up and add sufficient volume of elution buffer through the inlet fitting to cover the pleated membrane. Replace the inlet cap.
- 12.2.6.2.6** Return the capsule to the shaker with the bleed valve positioned at the 4 o'clock position. Turn on the shaker and agitate the capsule for approximately 5 minutes.
- 12.2.6.2.7** Remove the filter from the shaker, but leave the elution buffer in the capsule. Re-clamp the capsule to the shaker at the 8 o'clock position. Turn on the shaker and agitate the capsule for a final 5 minutes.
- 12.2.6.2.8** Remove the filter from the shaker and pour the contents into the 250-mL centrifuge tube. Rinse down the inside of the capsule filter walls with reagent water or elution buffer using a squirt bottle inserted in the inlet end of the capsule. Invert the capsule filter over the centrifuge tube and ensure that as much of the eluate as possible has been transferred.

12.2.7 Proceed to Section 13.0 for concentration and separation (purification).

- 12.3** Sample filtration using the Filta-Max™ foam filter. This procedure was validated using 50-L sample volumes. Alternate sample volumes may be used, provided the laboratory demonstrates acceptable performance on initial and ongoing spiked reagent water and source water samples (Section 9.1.2).

NOTE: The filtration procedures specified in Sections 12.3.1.2 - 12.3.1.6.3 are specific to laboratory filtration of a bulk sample. These procedures may require modification if samples will be filtered in the field.

12.3.1 Filtration

12.3.1.1 Flow rate adjustment

- 12.3.1.1.1** Connect the sampling system, minus the filter housing, to a carboy filled with reagent water.
- 12.3.1.1.2** Place the peristaltic pump upstream of the filter housing.

- 12.3.1.1.3** Turn on the pump and adjust the flow rate to 1 to 4 L per minute.

NOTE: A head pressure of 0.5 bar (7.5 psi) is required to create flow through the filter, and the recommended pressure of 5 bar (75 psi) should produce the flow rate of 3 to 4 L per minute. The maximum operating pressure of 8 bar (120 psi) should not be exceeded.

- 12.3.1.1.4** Allow 2 to 10 L of reagent water to flush the system. Adjust the pump speed as necessary during this period. Turn off the pump when the flow rate has been adjusted.
- 12.3.1.2** Place filter module into the filter housing and secure lid, hand tighten housings, apply gentle pressure to create the seal between the module and the ‘O’ rings in the base and the lid of the housing. Excessive tightening is not necessary, and may shorten the life of the ‘O’ rings. Tools may be used to tighten housing to the alignment marks (refer to manufacturer’s instructions). ‘O’ rings should be lightly greased before use (refer to manufacturer’s instructions).
- 12.3.1.3** Install the filter housing in the line, securing the inlet and outlet ends with the appropriate clamps/fittings. Verify that the filter housing is installed so that the end closest to the screw top cap is the inlet and the opposite end is the outlet.
- 12.3.1.4** Record the sample number, sample turbidity (if not provided with the field sample), and the name of the analyst filtering the sample on a bench sheet.
- 12.3.1.5** Filtration
- 12.3.1.5.1** Connect the sampling system to the field carboy of sample water, or transfer the sample water to the laboratory carboy used in Section 12.3.1.1.1. If the sample will be filtered from a field carboy, a spigot can be used with the carboy to facilitate sample filtration.

NOTE: If the bulk field sample is transferred to a laboratory carboy, the laboratory carboy must be cleaned and disinfected before it is used with another field sample.

- 12.3.1.5.2** Place the drain end of the sampling system tubing into an empty graduated container with a capacity greater than or equal to the volume to be filtered. This container will be used to determine the sample volume filtered. Alternately, connect a flow meter downstream of the filter, and record the initial meter reading.
- 12.3.1.5.3** Allow the carboy discharge tube and filter housing to fill with sample water. Turn on the pump to start water flowing through the filter. Verify that the flow rate is between 1 and 4 L per min.
- 12.3.1.5.4** After all of the sample has passed through the filter, turn off the pump. Allow the pressure to decrease until flow stops.
- 12.3.1.6** Disassembly
- 12.3.1.6.1** Disconnect the inlet end of the filter housing assembly while maintaining the level of the inlet fitting above the level of the outlet fitting to prevent backwashing and the loss of oocysts and cysts from the filter. Restart the

pump and allow as much water to drain as possible. Turn off the pump.

12.3.1.6.2 Based on the water level in the graduated container or the meter reading, record the volume filtered on a bench sheet to the nearest quarter liter.

12.3.1.6.3 Loosen the outlet fitting, the filter housing should be sealed with rubber plugs.

NOTE: *Filters should be prevented from drying out, as this can impair their ability to expand when decompressed.*

12.3.2 Elution

12.3.2.1 The filter is eluted to wash the oocysts from the filter. This can be accomplished using the Filta-Max™ wash station, which moves a plunger up and down a tube containing the filter and eluting solution (Section 12.3.2.2), or a stomacher, which uses paddles to agitate the stomacher bag containing the foam filter in the eluting solution (Section 12.3.2.3). If the Filta-Max™ automatic wash station is used please see the manufacturer's operator's guide for instructions on its use.

12.3.2.2 Filta-Max™ wash station elution procedure

12.3.2.2.1 First wash

- (a) Detach the removable plunger head using the tool provided, and remove the splash guard.
- (b) Place the filter membrane flat in the concentrator base with the rough side up. Locate the concentrator base in the jaws of the wash station and screw on the concentrator tube (the longer of the two tubes), creating a tight seal at the membrane. Take the assembled concentrator out of the jaws and place on the bench.
- (c) Replace the splash guard and temporarily secure it at least 15 cm above the end of the rack. Secure the plunger head with the tool provided ensuring that the lever is fully locked down.
- (d) Remove the filter module from the filter housing or transportation container. Pour excess liquid into the assembled concentrator, then rinse the housing or container with PBST and add the rinse to the concentrator tube. Screw the filter module onto the base of the plunger. Locate the elution tube base in the jaws of the wash station and screw the elution tube (the shorter of the two tubes) firmly in place.
- (e) Pull the plunger down until the filter module sits at the bottom of the elution tube; the locking pin (at the top left of the wash station) should "click" to lock the plunger in position.
- (f) Remove the filter module bolt by turning the adapted allen key (provided) in a clockwise direction (as seen from above). Attach the steel tube to the elution tube base.

- (g) Add 600 mL of PBST to the assembled concentrator. If more than 50 mL of liquid has been recovered from the shipped filter module, reduce the volume of PBST accordingly. Screw the concentrator tube onto the base beneath the elution tube. Release the locking pin.

NOTE: *Gentle pressure on the lever, coupled with a pulling action on the locking pin should enable the pin to be easily released.*

- (h) Wash the foam disks by moving the plunger up and down 20 times. Gentle movements of the plunger are recommended to avoid generating excess foam.

NOTE: *The plunger has an upper movement limit during the wash process to prevent it popping out of the top of the chamber.*

- (i) Detach the concentrator and hold it such that the stainless steel tube is just above the level of the liquid. Purge the remaining liquid from the elution tube by moving the plunger up and down 5 times, then lock the plunger in place. To prevent drips, place the plug provided in the end of the steel tube.
- (j) Prior to the second wash the eluate from the first wash can be concentrated using the Filta-Max™ apparatus according to Section 12.3.3.2.1 or the eluate can be decanted into a 2-L pooling beaker and set aside.

13.3.2.2.2

Second wash

- (a) Add an additional 600 mL of PBST to the concentrator module, remove the plug from the end of the steel tube and screw the concentrator tube back onto the elution module base. Release the locking pin.
- (b) Wash the foam disks by moving the plunger up and down 10 times. Gentle movements of the plunger are recommended to avoid generating excess foam.
- (c) The eluate can be concentrated using the Filta-Max™ apparatus according to Section 12.3.3.2.2 or the eluate can be decanted into the 2-L pooling beaker containing the eluate from the first wash and concentrated using centrifugation, as described in Section 12.3.3.3.

12.3.2.3

Stomacher elution procedure

12.3.2.3.1

First wash

- (a) Place the filter module in the stomacher bag then use the allen key to remove the bolt from the filter module, allowing the rings to expand. Remove the end caps from the stomacher bag and rinse with PBST into the stomacher bag.

(b) Add 600 mL of PBST to stomacher bag containing the filter pads. Place bag in stomacher and wash for 5 minutes on a normal setting.

(c) Remove the bag from the stomacher and decant the eluate into a 2-L pooling beaker.

12.3.2.3.2

Second wash

(a) Add a second 600-mL aliquot of PBST to the stomacher bag. Place bag in stomacher and wash for 5 minutes on a normal setting. Remove the bag from the stomacher and decant the eluate from the stomacher bag into the 2-L pooling beaker. Wring the stomacher bag by hand to remove eluate from the foam filter and add to the pooling beaker. Remove the foam filter from the bag and using a squirt bottle, rinse the stomacher bag with reagent water and add the rinse to the pooling beaker.

(b) Proceed to concentration (Section 12.3.3).

12.3.3 Concentration

12.3.3.1

The eluate can be concentrated using the Filta-Max™ concentrator apparatus, which pulls most of the eluate through a membrane filter leaving the oocysts concentrated in a small volume of the remaining eluting solution (Section 12.3.2), or by directly centrifuging all of the eluting solution used to wash the filter (Section 12.3.2.3).

12.3.3.2

The Filta-Max™ concentrator procedure

12.3.3.2.1

Concentration of first wash

- (a) If the stomacher was used to elute the sample (Section 12.3.2.3), transfer 600 mL of eluate from the pooling beaker to the concentrator tube. Otherwise proceed to Step (b).
- (b) Stand the concentrator tube on a magnetic stirring plate and attach the lid (with magnetic stirrer bar). Connect the waste bottle trap and hand or electric vacuum pump to the valve on the concentrator base. Begin stirring and open the tap. Increase the vacuum using the hand pump.

NOTE: *The force of the vacuum should not exceed 30 cmHg.*

- (c) Allow the liquid to drain until it is approximately level with the middle of the stirrer bar then close the valve. Remove the magnetic stirrer, and rinse it with PBST or distilled water to recover all oocysts. Decant the concentrate into a 50-mL tube, then rinse the sides of the concentration tube and add the rinsate to the 50-mL tube.

12.3.3.2.2

Concentration of second wash

- (a) If the stomacher was used to elute the sample (Section 12.3.2.3), transfer the remaining 600 mL of eluate from the pooling beaker to the concentrator tube. Otherwise proceed to Step (b).

- (b) Add the concentrate, in the 50-mL tube, retained from the first concentration (Section 12.3.3.2.1 (c)) to the 600 mL of eluate from the second wash, then repeat concentration steps from Sections 12.3.3.2.1 (b) and 12.3.3.2.1 (c). The final sample can be poured into the same 50-mL tube used to retain the first concentrate. Rinse the sides of the concentrator tube with PBST and add the rinse to the 50-mL tube.
- (c) Remove the magnetic stirrer. Insert the empty concentrator module into the jaws of the wash station and twist off the concentrator tube.
- (d) Transfer the membrane from the concentrator base to the bag provided using membrane forceps.

12.3.3.2.3

Membrane elution. The membrane can be washed manually or using a stomacher:

- **Manual wash.** Add 5 mL of PBST to the bag containing the membrane. Rub the surface of the membrane through the bag until the membrane appears clean. Using a pipette, transfer the eluate to a 50-mL tube. Repeat the membrane wash with another 5 mL of PBST and transfer the eluate to the 50-mL tube. (Optional: Perform a third wash using another 5 mL of PBST, by hand-kneading an additional minute or placing the bag on a flat-headed vortexer and vortexing for one minute. Transfer the eluate to the 50-mL tube.)

NOTE: Mark the bag with an “X” to note which side of the membrane has the oocysts to encourage the hand-kneading to focus on the appropriate side of the membrane.

- **Stomacher wash.** Add 5 mL of PBST to the bag containing the membrane. Place the bag containing the membrane into a small stomacher and stomach for 3 minutes. Using a pipette transfer the eluate to a 50-mL tube. Repeat the wash two times using the stomacher and 5-mL aliquots of PBST. (Optional: Perform a fourth wash using another 5 mL of PBST, by hand-kneading an additional minute or placing the bag on a flat-headed vortexer and vortexing for one minute. Transfer the eluate to the 50-mL tube.)

12.3.3.2.4

If the membrane filter clogs before concentration is complete, there are two possible options for completion of concentration. One option is replacing the membrane as often as necessary. Filter membranes may be placed smooth side up during the second concentration step. Another option is concentrating the remaining eluate using centrifugation. Both options are provided below.

- **Using multiple membranes.** Disassemble the concentrator tube and pour any remaining eluate back into the pooling beaker. Remove the membrane using membrane forceps, placing it in the bag provided. Place a new membrane in the concentrator

tube smooth side up, reassemble, return the eluate to the concentrator tube, rinse the pooling beaker and add rinse to the eluate, and continue the concentration. Replace the membrane as often as necessary.

- **Centrifuging remaining volume.** Decant the remaining eluate into a 2-L pooling beaker. Rinse the sides of the concentrator tube and add to the pooling beaker. Remove the filter membrane and place it in the bag provided. Wash the membrane as described in Section 12.3.3.2.3, then concentrate the sample as described in Section 12.3.3.3.1.

12.3.3.3 If the Filta-Max™ concentrator is not used for sample concentration, or if the membrane filter clogs before sample concentration is complete, then the procedures described in Section 12.3.3.3.1 should be used to concentrate the sample. If less than 50 mL of concentrate has been generated, the sample can be further concentrated, as described in Section 12.3.3.3.2, to reduce the volume of sample to be processed through IMS.

NOTE: The volume must not be reduced to less than 5 mL above the packed pellet. The maximum amount of pellet that should be processed through IMS is 0.5 mL. If the packed pellet is greater than 0.5 mL then the pellet may be subsampled as described in Section 13.2.4.

12.3.3.3.1 Centrifugation of greater than 50 mL of eluate

- Decant the eluate from the 2-L pooling beaker into 250-mL conical centrifuge tubes. Make sure that the centrifuge tubes are balanced.
- Centrifuge the 250-mL centrifuge tubes containing the eluate at $1500 \times G$ for 15 minutes. Allow the centrifuge to coast to a stop.
- Using a Pasteur pipette, carefully aspirate off the supernatant to 5 mL above the pellet. If the sample is reagent water (e.g. initial or ongoing precision and recovery sample) extra care must be taken to avoid aspirating oocysts and cysts during this step.
- Vortex each 250-mL tube vigorously until pellet is completely resuspended. Swirl the centrifuge tube gently to reduce any foaming after vortexing. Combine the contents of each 250-mL centrifuge tube into a 50-mL centrifuge tube. Rinse each of the 250-mL centrifuge tubes with PBST and add the rinse to the 50-mL tube.
- Proceed to Section 12.3.3.3.2.

12.3.3.3.2 Centrifugation of less than 50 mL of eluate

- Centrifuge the 50-mL centrifuge tube containing the combined concentrate at $1500 \times G$ for 15 minutes. Allow the centrifuge to coast to a stop. Record the initial pellet volume (volume of solids) and the date

and time that concentration was completed on a bench sheet.

(b) Proceed to Section 13.0 for concentration and separation (purification).

12.3.4 Maintenance and cleaning

12.3.4.1 Maintenance of O-rings

12.3.4.1.1 Check all rubber O-rings for wear or deterioration prior to each use and replace as necessary.

12.3.4.1.2 Lubricate the plunger head O-ring inside and out with silicon before each use.

12.3.4.1.3 Lubricate all other O-rings (concentrator tube set, filter housing) regularly in order to preserve their condition.

12.3.4.2 Cleaning

12.3.4.2.1 All components of the Filta-Max™ system can be cleaned using warm water and laboratory detergent. After washing, rinse all components with oocyst and cyst free reagent water and dry them. All O-rings should be re-lubricated. Alternatively a mild (40°C) dishwasher cycle without bleach or rinse aid can be used.

12.3.4.2.2 To wash the detachable plunger head slide the locking pin out and wash the plunger head and locking pin in warm water and laboratory detergent. Rinse the plunger head and locking pin with oocyst and cyst free reagent water and dry. Lightly lubricate the locking pin and re-assemble the plunger head.

13.0 Sample Concentration and Separation (Purification)

13.1 During concentration and separation, the filter eluate is concentrated through centrifugation, and the oocysts and cysts in the sample are separated from other particulates through immunomagnetic separation (IMS). Alternate procedures and products may be used if the laboratory first demonstrates equivalent or superior performance as per Section 9.1.2.

13.2 Adjustment of pellet volume

13.2.1 Centrifuge the 250-mL centrifuge tube containing the capsule filter eluate at $1500 \times G$ for 15 minutes. Allow the centrifuge to coast to a stop—do not use the brake. Record the pellet volume (volume of solids) on the bench sheet.

NOTE: Recoveries may be improved if centrifugation force is increased to $2000 \times G$. However, do not use this higher force if the sample contains sand or other gritty material that may degrade the condition of any oocysts and/or cysts in the sample.

13.2.2 Using a Pasteur pipette, carefully aspirate the supernatant to 5 mL above the pellet. Extra care must be taken to avoid aspirating oocysts and cysts during this step, particularly if the sample is reagent water (e.g. initial or ongoing precision and recovery sample).

13.2.3 If the packed pellet volume is ≤ 0.5 mL, vortex the tube vigorously until pellet is completely resuspended. Swirl the centrifuge tube gently to reduce any foaming after vortexing. Record the resuspended pellet volume on the bench sheet. Proceed to Section 13.3.

NOTE: *Extra care must be taken with samples containing sand or other gritty material when vortexing to ensure that the condition of any oocysts and/or cysts in the sample is not compromised.*

13.2.4 If the packed pellet volume is > 0.5 mL, the concentrate ~~needs to must~~ be separated into multiple subsamples (a subsample is equivalent to no greater than 0.5 mL of packed pellet material, the recommended maximum amount of particulate material to process through the subsequent purification and examination steps in the method). Use the following formula to determine the total volume required in the centrifuge tube before separating the concentrate into two or more subsamples:

$$\text{total volume (mL) required} = \frac{\text{pellet volume}}{0.5 \text{ mL}} \times 5 \text{ mL}$$

(For example, if the packed pellet volume is 1.2 mL, the total volume required is 12 mL.) Add reagent water to the centrifuge tube to bring the total volume to the level calculated above. ~~Vortex the tube vigorously for 10 to 15 seconds to completely resuspend the pellet. Record the resuspended pellet volume on the bench sheet.~~

NOTE: *Extra care must be taken with samples containing sand or other gritty material when vortexing to ensure that the condition of any oocysts in the sample is not compromised.*

13.2.4.1 **Analysis of entire sample.** If analysis of the entire sample is required, determine the number of subsamples to be processed independently through the remainder of the method:

13.2.4.1.1 **Calculate number of subsamples:** Divide the total volume in the centrifuge tube by 5 mL and round up to the nearest integer (for example, if the resuspended volume in Section 13.2.4 is 12 mL, then the number of subsamples would be 12 mL / 5 mL = 2.4, rounded = 3 subsamples).

13.2.4.1.2 **Determine volume of resuspended concentrate per subsample.** Divide the total volume in the centrifuge tube by the calculated number of subsamples (for example, if the resuspended volume in Section 13.2.4 is 12 mL, then the volume to use for each subsample = 12 mL / 3 subsamples = 4 mL).

13.2.4.1.3 **Process subsamples through IMS.** ~~Vortex the tube vigorously for 10 to 15 seconds to completely resuspend the pellet. Record the resuspended pellet volume on the bench sheet.~~ Proceed **immediately** to Section 13.3, and transfer aliquots of the resuspended concentrate equivalent to the volume in the previous step to multiple, flat-sided sample tubes in Section 13.3.2.1. Process the sample as multiple, independent subsamples from Section 13.3 onward, including the preparation and examination of separate slides for each aliquot. Record the volume of resuspended concentrate transferred to IMS on the bench sheet (this will be equal to the volume recorded in Section 13.2.4). Also record the number of

subsamples processed independently through the method on the bench sheet.

- 13.2.4.2 Analysis of partial sample.** If not all of the concentrate will be examined, **vortex the tube vigorously for 10 to 15 seconds to completely resuspend the pellet. Record the resuspended pellet volume on the bench sheet.** Proceed **immediately** to Section 13.3, and transfer one or more 5-mL aliquots of the resuspended concentrate to one or more flat-sided sample tubes in Section 13.3.2.1. Record the volume of resuspended concentrate transferred to IMS on the bench sheet. To determine the volume analyzed, calculate the percent of the concentrate examined using the following formula:

$$\text{percent examined} = \frac{\text{total volume of resuspended concentrate transferred to IMS}}{\text{total volume of resuspended concentrate in Section 13.2.4}} \times 100\%$$

Then multiply the volume filtered (Section 12.2.5.2) by this percentage to determine the volume analyzed.

13.3 IMS procedure (adapted from Reference 20.11)

NOTE: The IMS procedure should be performed on a bench top with all materials at room temperature, ranging from 15°C to 25°C.

13.3.1 Preparation and addition of reagents

- 13.3.1.1** Prepare a 1X dilution of SL-buffer-A from the 10X SL-buffer-A (clear, colorless solution) supplied. Use reagent water (demineralized; Section 7.3) as the diluent. For every 1 mL of 1X SL-buffer-A required, take 100 µL of 10X SL-buffer-A and make up to 1 mL with the diluent water. A volume of 1.5 mL of 1X SL-buffer-A will be required per sample or subsample on which the Dynal IMS procedure is performed.
- 13.3.1.2** For each sample or subsample (Section 13.2) to be processed through IMS, add 1 mL of the 10X SL-buffer-A (supplied—not the diluted 1X SL-buffer-A) to a flat-sided tube (Section 6.5.4).
- 13.3.1.3** For each subsample, add 1 mL of the 10X SL-buffer-B (supplied—magenta solution) to the flat-sided tube containing the 10X SL-buffer-A.

13.3.2 Oocyst and cyst capture

- 13.3.2.1** Use a graduated, 10-mL pipette that has been pre-rinsed with elution buffer to transfer the water sample concentrate from Section 13.2 to the flat-sided tube(s) containing the SL-buffers. If all of the concentrate is used, rinse the centrifuge tube twice with reagent water and add the rinsate to the flat-sided tube containing the concentrate (or to the tube containing the first subsample, if multiple subsamples will be processed). Each of the two rinses should be half the volume needed to bring the volume in the flat-sided sample tube to 10 mL (**including the buffers added in Sections 13.3.1.2 and 13.3.1.3**). (For example, if the tube contained 1 mL of SL-buffer-A and 1 mL of SL-buffer-B, and 5 mL of sample was transferred after resuspension of the pellet, **for a total of 7 mL**, the centrifuge tube would be rinsed twice with **1.5 mL** of reagent water to bring the total volume in the flat-sided tube to 10 mL.) Visually inspect the centrifuge tube after completing the transfer to ensure that no concentrate remains. If multiple subsamples will be processed, bring the

- p>volume in the remaining flat-sided tubes to 10 mL with reagent water. Label the flat-sided tube(s) with the sample number (and subsample letters).
- 13.3.2.2** Vortex the Dynabeads®Crypto-Combo vial from the IMS kit for approximately 10 seconds to suspend the beads. Ensure that the beads are fully resuspended by inverting the sample tube and making sure that there is no residual pellet at the bottom.
 - 13.3.2.3** Add 100 µL of the resuspended Dynabeads®Crypto-Combo (Section 13.3.2.2) to the sample tube(s) containing the water sample concentrate and SL-buffer.
 - 13.3.2.4** Vortex the Dynabeads®Giardia-Combo vial from the IMS kit for approximately 10 seconds to suspend the beads. Ensure that the beads are fully resuspended by inverting the tube and making sure that there is no residual pellet at the bottom.
 - 13.3.2.5** Add 100 µL of the resuspended Dynabeads®Giardia-Combo (Section 13.3.2.4) to the sample tube(s) containing the water sample concentrate, Dynabeads®Crypto-Combo, and SL-buffer.
 - 13.3.2.6** Affix the sample tube(s) to a rotating mixer and rotate at approximately 18 rpm for 1 hour at room temperature.
 - 13.3.2.7** After rotating for 1 hour, remove each sample tube from the mixer and place the tube in the magnetic particle concentrator (MPC-1) with flat side of the tube toward the magnet.
 - 13.3.2.8** Without removing the sample tube from the MPC-1, place the magnet side of the MPC-1 downwards, so the tube is horizontal and the flat side of the tube is facing down.
 - 13.3.2.9** Gently rock the sample tube by hand end-to-end through approximately 90°, tilting the cap-end and base-end of the tube up and down in turn. Continue the tilting action for 2 minutes with approximately one tilt per second.
 - 13.3.2.10** Ensure that the tilting action is continued throughout this period to prevent binding of low-mass, magnetic or magnetizable material. If the sample in the MPC-1 is allowed to stand motionless for more than 10 seconds, **remove the flat-sided tube from the MPC-1, shake the tube to resuspend all material, replace the sample tube in the MPC-1 and repeat Section 13.3.2.9 before continuing to Section 13.3.2.11.**
 - 13.3.2.11** Return the MPC-1 to the upright position, sample tube vertical, with cap at top. Immediately remove the cap and, keeping the flat side of the tube on top, pour off all of the supernatant from the tube held in the MPC-1 into a suitable container. Do not shake the tube and do not remove the tube from MPC-1 during this step. **Allow more supernatant to settle; aspirate additional supernatant with pipette.**
 - 13.3.2.12** Remove the sample tube from the MPC-1 and resuspend the sample in ~~± mL~~**0.5 mL** 1X SL-buffer-A (prepared from 10X SL-buffer-A stock—supplied). Mix very gently to resuspend all material in the tube. Do not vortex.
 - 13.3.2.13** Quantitatively transfer (transfer followed by two rinses) all the liquid from the sample tube to a labeled, 1.5-mL microcentrifuge tube. Use ~~± mL~~**0.5 mL of 1X SL-buffer-A to perform the first rinse and 0.5 mL of reagent water for the second rinse. Liberally rinse down the sides of the Leighton tube before transferring and transfer the rinsate to the**

~~microcentrifuge tube.~~ Allow the flat-sided sample tube to sit for a minimum of 1 minute after transfer of the second rinse volume, then use a pipette to collect any residual volume that drips down to the bottom of the tube to ensure that as much sample volume is recovered as possible. Ensure that all of the liquid and beads are transferred.

- 13.3.2.14** Place the microcentrifuge tube into the second magnetic particle concentrator (MPC-M), with its magnetic strip in place.
- 13.3.2.15** Without removing the microcentrifuge tube from MPC-M, gently rock/roll the tube through 180° by hand. Continue for approximately 1 minute with approximately one 180° roll/rock per second. At the end of this step, the beads should produce a distinct brown dot at the back of the tube.
- 13.3.2.16** Immediately aspirate the supernatant from the tube and cap held in the MPC-M. If more than one sample is being processed, conduct three 90° rock/roll actions before removing the supernatant from each tube. Take care not to disturb the material attached to the wall of the tube adjacent to the magnet. *Do not shake the tube. Do not remove the tube from MPC-M while conducting these steps.*

13.3.3 Dissociation of beads/oocyst/cyst complex

NOTE: Two acid dissociations are required.

- 13.3.3.1** Remove the magnetic strip from the MPC-M.
- 13.3.3.2** Add 50 µL of 0.1 N HCl, then vortex at the highest setting for approximately 50 seconds.

NOTE: The laboratory ~~should~~ must use 0.1-N standards purchased directly from a vendor, rather than adjusting the normality in-house.

- 13.3.3.3** Place the tube in the MPC-M without the magnetic strip in place and allow to stand in a vertical position for at least 10 minutes at room temperature.
- 13.3.3.4** Vortex vigorously for approximately 30 seconds.
- 13.3.3.5** Ensure that all of the sample is at the base of the tube. Place the microcentrifuge tube in the MPC-M.
- 13.3.3.6** Replace magnetic strip in MPC-M and allow the tube to stand undisturbed for a minimum of 10 seconds.
- 13.3.3.7** Prepare a well slide for sample screening and label the slide.
- 13.3.3.8** Add 5 µL of 1.0 N NaOH to the sample wells of two well slides (add 10 µL to the sample well of one well slide if the volume from the two required dissociations will be added to the same slide).

NOTE: The laboratory ~~should~~ must use 1.0-N standards purchased directly from a vendor rather than adjusting the normality in-house.

- 13.3.3.9** Without removing the microcentrifuge tube from the MPC-M, transfer all of the sample from the microcentrifuge tube in the MPC-M to the sample well with the NaOH. Do not disturb the beads at the back wall of the tube. Ensure that all of the fluid is transferred.
- 13.3.3.10** Do not discard the beads or microcentrifuge tube after transferring the volume from the first acid dissociation to the well slide. Perform the steps in Sections 13.3.3.1 through 13.3.3.9 a second time. The volume

from the second dissociation can be added to the slide containing the volume from the first dissociation, or can be applied to a second slide.

NOTE: ~~If one slide is used, exert extra care when using The wells on Dynal Spot-On slides are likely to be too small to accommodate the volumes from both dissociations.~~

13.3.3.11 Record the date and time the purified sample was applied to the slide(s).

13.3.3.12 Air-dry the sample on the well slide(s). Because temperature and humidity varyies from laboratory to laboratory, no minimum time is specified. However, the laboratory must take care to ensure that the sample has dried completely before staining to prevent losses during the rinse steps. A slide warmer set at 35°C to 42°C also can be used.

13.3.4 Tips for minimizing carry-over of debris onto microscope slides after IMS

- Make sure the resuspended pellet is fully homogenized before placing the tube in the MPC-1 or MPC-M to avoid trapping "clumps" or a dirty layer between the beads and the side of the tube.
- When using the MPC-1 magnet, make sure that the tube is snugged flat against the magnet. Push the tube flat if necessary. Sometimes the magnet is not flush with the outside of the holder and, therefore, the attraction between the beads and the magnet is not as strong as it should be. However, it can be difficult to determine this if you do not have more than one MPC-1 to make comparisons.
- After the supernatant has been poured off at Section 13.3.2.11, leave the tube in the MPC-1 and allow time for any supernatant remaining in the tube to settle down to the bottom. Then aspirate the settled supernatant and associated particles from the bottom of the tube. The same can be done at Section 13.3.2.16 with the microcentrifuge tube.
- An additional rinse can also be performed at Section 13.3.2.11. After the supernatant has been poured off and any settled material is aspirated off the bottom, leave the tube in the MPC-1 and add an additional 10 mL of reagent water or PBS to the tube and repeat Sections 13.3.2.9 and 13.3.2.11. Although labs have reported successfully using this technique to reduce carryover, because the attraction between the MPC-1 and the beads is not as great as the attraction between the MPC-M and the beads, the chances would be greater for loss of cysts and oocysts doing the rinse at this step instead of at Section 13.3.2.16.
- After the supernatant has been aspirated from the tube at Section 13.3.2.16, add 0.1 mL of PBS, remove the tube from the MPC-M, and resuspend. Repeat Sections 13.3.2.15 and 13.3.2.16.
- Use a slide with the largest diameter well available to spread out the sample as much as possible.

14.0 Sample Staining

NOTE: The sample must be stained within 72 hours of application of the purified sample to the slide.

14.1 Prepare positive and negative controls.

14.1.1 For the positive control, pipette 10 µL of positive antigen or 200 to 400 intact oocysts and 200 to 400 cysts to the center of a well.

14.1.2 For the negative control, pipette 50 µL of 150 mM PBS (Section 7.6.4) into the center of a well and spread it over the well area with a pipette tip.

14.1.3 Air-dry the control slides (see Section 13.3.3.12 for guidance).

NOTE: If the laboratory has a large batch of slides that will be examined over several days, and is concerned that a single positive control may fade, due to multiple examinations, the laboratory should prepare multiple control slides with the batch of field slides and alternate between the positive controls when performing the positive control check.

~~14.2 Apply 50- μ L of absolute methanol to each well containing the dried sample and allow to air-dry for 3 to 5 minutes.~~

14.2 Follow manufacturer's instructions in applying stain to slides.

14.3 Place the slides in a humid chamber in the dark and incubate at room temperature for approximately 30 minutes. The humid chamber consists of a tightly sealed plastic container containing damp paper towels on top of which the slides are placed.

14.4 Remove slides from humid chamber and allow condensation to evaporate, if present.

14.5 Apply one drop of wash buffer (prepared according to the manufacturer's instructions [Section 7.6]) to each well. Tilt each slide on a clean paper towel, long edge down. Gently aspirate the excess detection reagent from below the well using a clean Pasteur pipette or absorb with paper towel or other absorbent material placed at edge of slide. Avoid disturbing the sample.

NOTE: If using the Merifluor stain (Section 7.6.1), do not allow slides to dry completely.

14.6 Apply 50 μ L of 4',6-diamidino-2-phenylindole (DAPI) staining solution (Section 7.7.2) to each well. Allow to stand at room temperature for a minimum of 1 minute. (The solution concentration may be increased up to 1 μ g/mL if fading/diffusion of DAPI staining is encountered, but the staining solution must be tested first on expendable environmental samples to confirm that staining intensity is appropriate.)

14.7 Apply one drop of wash buffer (prepared according to the manufacturer's instructions [Section 7.6]) to each well. Tilt each slide on a clean paper towel, long edge down. Gently aspirate the excess DAPI staining solution from below the well using a clean Pasteur pipette or absorb with paper towel or other absorbent material placed at edge of slide. Avoid disturbing the sample.

NOTE: If using the Merifluor stain (Section 7.6.1), do not allow slides to dry completely.

14.8 Add mounting medium (Section 7.8) to each well.

14.9 Apply a cover slip. Use a tissue to remove excess mounting fluid from the edges of the coverslip. Seal the edges of the coverslip onto the slide using clear nail polish.

14.10 Record the date and time that staining was completed on the bench sheet. If slides will not be read immediately, store in a humid chamber in the dark at ~~0°C to~~ $< 8^{\circ}\text{C}$ (but not frozen) until ready for examination.

15.0 Examination

NOTE: Although immunofluorescence assay (FA) and 4',6-diamidino-2-phenylindole (DAPI) and differential interference contrast (DIC) microscopy examination ~~and confirmation~~ should be performed immediately after staining is complete, laboratories have up to 7 days from completion of sample staining to complete the examination and confirmation of samples. However, if fading/diffusion of FITC or DAPI staining is noticed, the laboratory must reduce this holding time. In addition the laboratory may adjust the concentration of the DAPI staining solution (Sections 7.7.2) so that fading/diffusion does not occur.

15.1 Scanning technique: Scan each well in a systematic fashion. An up-and-down or a side-to-side scanning pattern may be used (Figure 4).

- 15.2** Examination using immunofluorescence assay (FA), 4',6-diamidino-2-phenylindole (DAPI) staining characteristics, and differential interference contrast (DIC) microscopy. The minimum magnification requirements for each type of examination are noted below.

NOTE: All *shape-characterization* and *size* measurements must be determined using 1000X magnification and reported to the nearest 0.5 μm .

Record examination results for *Cryptosporidium* oocysts on a *Cryptosporidium* **report examination results** form; record examination results for *Giardia* cysts on a *Giardia* **report examination results** form. All oocysts and cysts that meet the criteria specified in Sections 15.2.2 and 15.2.3, less atypical organisms specifically identified as non-target organisms by DIC or DAPI (e.g. possessing spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc), must be reported.

- 15.2.1** Positive and negative staining control. **Positive and negative staining controls must be acceptable.**

- 15.2.1.1** Each analyst must characterize a minimum of three *Cryptosporidium* oocysts and three *Giardia* cysts on the positive staining control slide before examining field sample slides. This characterization must be performed by each analyst during each microscope examination session. FITC examination must be conducted at a minimum of 200X total magnification, DAPI examination must be conducted at a minimum of 400X, and DIC examination **and size measurements** must be conducted at a minimum of 1000X. Size, shape, and DIC and DAPI characteristics of the three *Cryptosporidium* oocysts and *Giardia* cysts must be recorded by the analyst on a microscope log. The analyst also must indicate on each sample **report examination results** form whether the positive staining control was acceptable.
- 15.2.1.2** Examine the negative staining control to confirm that it does not contain any oocysts or cysts (Section 14.1). Indicate on each sample **report examination results** form whether the negative staining control was acceptable.
- 15.2.1.3** If the positive staining control contains oocysts and cysts within the expected range and at the appropriate fluorescence for both FA and DAPI, and the negative staining control does not contain any oocysts or cysts (Section 14.1), proceed to Sections 15.2.2 and 15.2.3.

- 15.2.2** Sample examination—*Cryptosporidium*

- 15.2.2.1** **FITC examination** (the analyst must use a minimum of 200X total magnification). Use epifluorescence to scan the entire well for apple-green fluorescence of oocyst and cyst shapes. When brilliant apple-green fluorescing ovoid or spherical objects 4 to 6 μm in diameter are observed with brightly highlighted edges, increase magnification to 400X and switch the microscope to the UV filter block for DAPI (Section 15.2.2.2), then to DIC (Section 15.2.2.3) **at 1000X**.
- 15.2.2.2** **DAPI examination** (the analyst must use a minimum of 400X total magnification). Using the UV filter block for DAPI, the object will exhibit one of the following characteristics:
- (a) Light blue internal staining (no distinct nuclei) with a green rim
 - (b) Intense blue internal staining
 - (c) Up to four distinct, sky-blue nuclei
- Record oocysts in category (a) as DAPI negative; record oocysts in categories (b) and (c) as DAPI positive.

- 15.2.2.3 DIC examination** (the analyst must use a minimum of 1000X total magnification [oil immersion lens]). Using DIC, look for external or internal morphological characteristics atypical of *Cryptosporidium* oocysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.) (adapted from Reference 20.7). If atypical structures are not observed, then categorize each apple-green fluorescing object as:
- (a) An empty *Cryptosporidium* oocyst
 - (b) A *Cryptosporidium* oocyst with amorphous structure
 - (c) A *Cryptosporidium* oocyst with internal structure (one to four sporozoites/oocyst)

Using 1000X total magnification, record the shape, measurements (to the nearest 0.5 μm), and number of sporozoites (if applicable) for each apple-green fluorescing object meeting the size and shape characteristics. Although not a defining characteristic, surface oocyst folds may be observed in some specimens.

NOTE: All measurements must be made at 1000X magnification.

15.2.3 Sample examination—*Giardia*

- 15.2.3.1 FITC examination** (the analyst must use a minimum of 200X total magnification). When brilliant apple-green fluorescing round to **oval** **ovoid** objects (8 - 18 μm long by 5 - 15 μm wide) are observed, increase magnification to 400X and switch the microscope to the UV filter block for DAPI (Section 15.2.3.2) then to DIC (Section 15.2.3.3) **at 1000X**.

- 15.2.3.2 DAPI examination** (the analyst must use a minimum of 400X total magnification). Using the UV filter block for DAPI, the object will exhibit one or more of the following characteristics:
- (a) Light blue internal staining (no distinct nuclei) and a green rim
 - (b) Intense blue internal staining
 - (c) Two to four sky-blue nuclei
- Record cysts in category (a) as DAPI negative; record cysts in categories (b) and (c) as DAPI positive.

- 15.2.3.3 DIC examination** (the analyst must use a minimum of 1000X total magnification [oil immersion lens]). Using DIC, look for external or internal morphological characteristics atypical of *Giardia* cysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.) (adapted from Reference 20.7). If atypical structures are not observed, then categorize each object meeting the criteria specified in Sections 15.2.3.1 - 15.2.3.3 as one of the following, based on DIC examination:
- (a) An empty *Giardia* cyst
 - (b) A *Giardia* cyst with amorphous structure
 - (c) A *Giardia* cyst with one type of internal structure (nuclei, median body, or axonemes), or
 - (d) A *Giardia* cyst with more than one type of internal structure

Using 1000X total magnification, record the shape, measurements (to the nearest 0.5 μm), and number of nuclei and presence of median body or axonemes (if applicable) for each apple-green fluorescing object meeting the size and shape characteristics.

NOTE: All measurements must be made at 1000X magnification.

15.2.4 Record the date and time that sample examination was completed on the **report examination results** form.

15.2.5 Report *Cryptosporidium* and *Giardia* concentrations as oocysts/L and cysts/L.

15.2.6 Record analyst name

16.0 Analysis of Complex Samples

16.1 Some samples may contain high levels (>1000/L) of oocysts and cysts and/or interfering organisms, substances, or materials. Some samples may clog the filter (Section 12.0); others will not allow separation of the oocysts and cysts from the retentate or eluate; and others may contain materials that preclude or confuse microscopic examination.

16.2 If the sample holding time has not been exceeded and a full-volume sample cannot be filtered, dilute an aliquot of sample with reagent water and filter this smaller aliquot (Section 12.0). This dilution must be recorded and reported with the results.

16.3 If the holding times for the sample and for microscopic examination of the cleaned up retentate/eluate have been exceeded, the site should be re-sampled. If this is not possible, the results should be qualified accordingly.

17.0 Method Performance

17.1 Method acceptance criteria are shown in Tables 3 and 4 in Section 21.0. The initial and ongoing precision and recovery criteria are based on the results of spiked reagent water samples analyzed during the Information Collection Rule Supplemental Surveys (Reference 20.8). The matrix spike and matrix spike duplicate criteria are based on spiked source water data generated during the interlaboratory validation study of Method 1623 involving 11 laboratories and 11 raw surface water matrices across the U.S. (Reference 20.10).

NOTE: Some sample matrices may prevent the MS acceptance criteria in Tables 3 and 4 to be met. An assessment of the distribution of MS recoveries across 430 MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 5.

18.0 Pollution Prevention

18.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

18.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials **that need to be discarded to be disposed**.

19.0 Waste Management

19.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required. An overview of these requirements can be found in the *Environmental Management Guide for Small Laboratories* (EPA 233-B-98-001).

19.2 Samples, reference materials, and equipment known or suspected to have viable oocysts or cysts attached or contained must be sterilized prior to disposal.

19.3 For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* and *Less is Better: Laboratory Chemical Management for Waste Reduction*, both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036.

20.0 References

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- 20.5** "Safety in Academic Chemistry Laboratories," ACS Committee on Chemical Safety (1979).
- 20.6** USEPA. *Manual for the Certification of Laboratories Analyzing Drinking Water: Criteria and Procedures; Quality Assurance; Fourth Edition*, EPA 815-B-97-001, Office of Ground Water and Drinking Water, U.S. Environmental Protection Agency, 26 West Martin Luther King Drive, Cincinnati, OH 45268 (1997).
- 20.7** *ICR Microbial Laboratory Manual*, EPA/600/R-95/178, National Exposure Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, 26 Martin Luther King Drive, Cincinnati, OH 45268 (1996).
- ~~**20.8** USEPA. *EPA Guide to Method Flexibility and Approval of EPA Water Methods*, EPA 821-D-96-004. Office of Water, Engineering and Analysis Division, Washington, DC 20460 (1996).~~
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- 20.9** "Envirochek™ Sampling Capsule," PN 32915, Gelman Sciences, 600 South Wagner Road, Ann Arbor, MI 48103-9019 (1996).
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- 20.12** USEPA. *Implementation and Results of the Information Collection Rule Supplemental Surveys*. EPA-815-R-01-003. Office of Water, Office of Ground Water and Drinking Water, Standards and Risk Management Division, Washington, DC (2001).
- 20.13** Connell, K., J. Scheller, K. Miller, and C.C. Rodgers, 2000. *Performance of Methods 1622 and 1623 in the ICR Supplemental Surveys*. Proceedings, American Water Works Association Water Quality Technology Conference, November 5 - 9, 2000, Salt Lake City, UT.

21.0 Tables and Figures

Table 1. Method Holding Times (See Section 8.2 for details)

Sample Processing Step	Maximum Allowable Time between Breaks <i>(Samples should be processed as soon as possible)</i>
Collection	
Filtration	
➤ Up to 96 hours are permitted between sample collection (if shipped to the laboratory as a bulk sample) or filtration (if filtered in the field) and initiation of elution	
Elution	These steps must be completed in 1 working day
Concentration	
Purification	
Application of purified sample to slide	
Drying of sample	
➤ Up to 72 hours are permitted from application of the purified sample to the slide to staining	
Staining	
➤ Up to 7 days are permitted between sample staining and examination	
Examination	

Table 2. Tier 1 and Tier 2 Validation/Equivalency Demonstration Requirements

Test	Description	Tier 1 modification ⁽¹⁾	Tier 2 modification ⁽²⁾
IPR (Section 9.4)	4 replicates of spiked reagent water	Required. Must be accompanied by a method blank.	Required per laboratory
Method blank (Section 9.6)	Unspiked reagent water	Required	Required per laboratory
MS (Section 9.5.1)	Spiked matrix water	Required on each water to which the modification will be applied and on every 20th sample of that water thereafter. Must be accompanied by an unspiked field sample collected at the same time as the MS sample	Not required
MS/MSD (Section 9.5)	2 replicates of spiked matrix water	Recommended, but not required. Must be accompanied by an unspiked field sample collected at the same time as the MS sample	Required per laboratory. Each laboratory must analyze a different water.

- (1) If a modification will be used only in one laboratory, these tests must be performed and the results must meet all of the QC acceptance criteria in the method (these tests also are required the first time a laboratory uses the validated version of the method)
- (2) If nationwide approval of a modification is sought for one type of water matrix (such as surface water), a minimum of 3 laboratories must perform the tests and the results from each lab individually must meet all QC acceptance criteria in the method. If more than 3 laboratories are used in a study, a minimum of 75% of the laboratories must meet all QC acceptance criteria.

NOTE: The initial precision and recovery and ongoing precision and recovery (OPR) acceptance criteria listed in Tables 3 and 4 are based on results from 293 *Cryptosporidium* OPR samples and 186 *Giardia* OPR samples analyzed by six laboratories during the Information Collection Rule Supplemental Surveys (Reference 20.13). The matrix spike acceptance criteria are based on data generated through interlaboratory validation of Method 1623 (Reference 20.11).

Table 3. Quality Control Acceptance Criteria for *Cryptosporidium*

Performance test	Section	Acceptance criteria
Initial precision and recovery	9.4	
Mean recovery (percent)	9.4.2	24 - 100
Precision (as maximum relative standard deviation)	9.4.2	55
Ongoing precision and recovery (percent)	9.7	11 - 100
Matrix spike/matrix spike duplicate (for method modifications)	9.5	
Mean recovery ^{1,2} (as percent)	9.5.2	13 - 111
Precision (as maximum relative percent difference)	9.5.2	61

(1) The acceptance criteria for mean MS/MSD recovery serves as the acceptance criteria for MS recovery during routine use of the method (Section 9.5.1).

(2) Some sample matrices may prevent the acceptance criteria from being met. An assessment of the distribution of MS recoveries from multiple MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 5.

Table 4. Quality Control Acceptance Criteria for *Giardia*

Performance test	Section	Acceptance criteria
Initial precision and recovery	9.4	
Mean recovery (percent)	9.4.2	24 - 100
Precision (as maximum relative standard deviation)	9.4.2	49
Ongoing precision and recovery (percent)	9.7	14 - 100
Matrix spike/matrix spike duplicate (for method modifications)	9.5	
Mean recovery* (as percent)	9.5.2	15 - 118
Precision (as maximum relative percent difference)	9.5.2	30

(1) The acceptance criteria for mean MS/MSD recovery serves as the acceptance criteria for MS recovery during routine use of the method (Section 9.5.1).

(2) Some sample matrices may prevent the acceptance criteria from being met. An assessment of the distribution of MS recoveries across multiple MS samples from 87 sites during the ICR Supplemental Surveys is provided in Table 5.

Table 5. Distribution of Matrix Spike Recoveries from Multiple Samples Collected from 87 Source Waters During the ICR Supplemental Surveys (Adapted from Reference 20.14)

MS Recovery Range	Percent of 430 <i>Cryptosporidium</i> MS Samples in Recovery Range	Percent of 270 <i>Giardia</i> MS Samples in Recovery Range
<10%	6.7%	5.2%
>10% - 20%	6.3%	4.8%
>20% - 30%	14.9%	7.0%
>30% - 40%	14.2%	8.5%
>40% - 50%	18.4%	17.4%
>50% - 60%	17.4%	16.3%
>60% - 70%	11.2%	16.7%
>70% - 80%	8.4%	14.1%
>80% - 90%	2.3%	6.3%
>90%	0.2%	3.7%

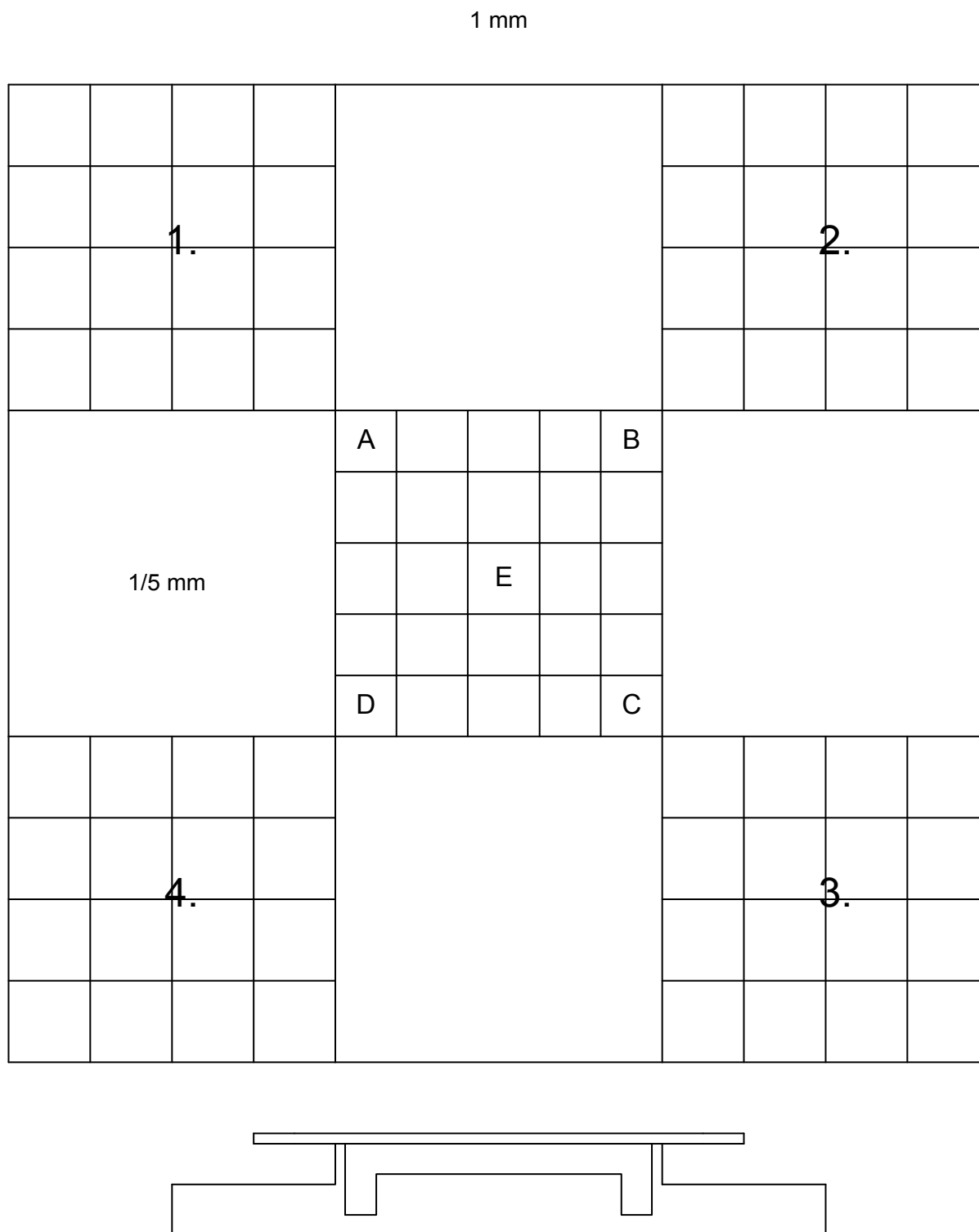


Figure 1. Hemacytometer Platform Ruling. Squares 1, 2, 3, and 4 are used to count stock suspensions of *Cryptosporidium* oocysts and *Giardia* cysts (after Miale, 1967)

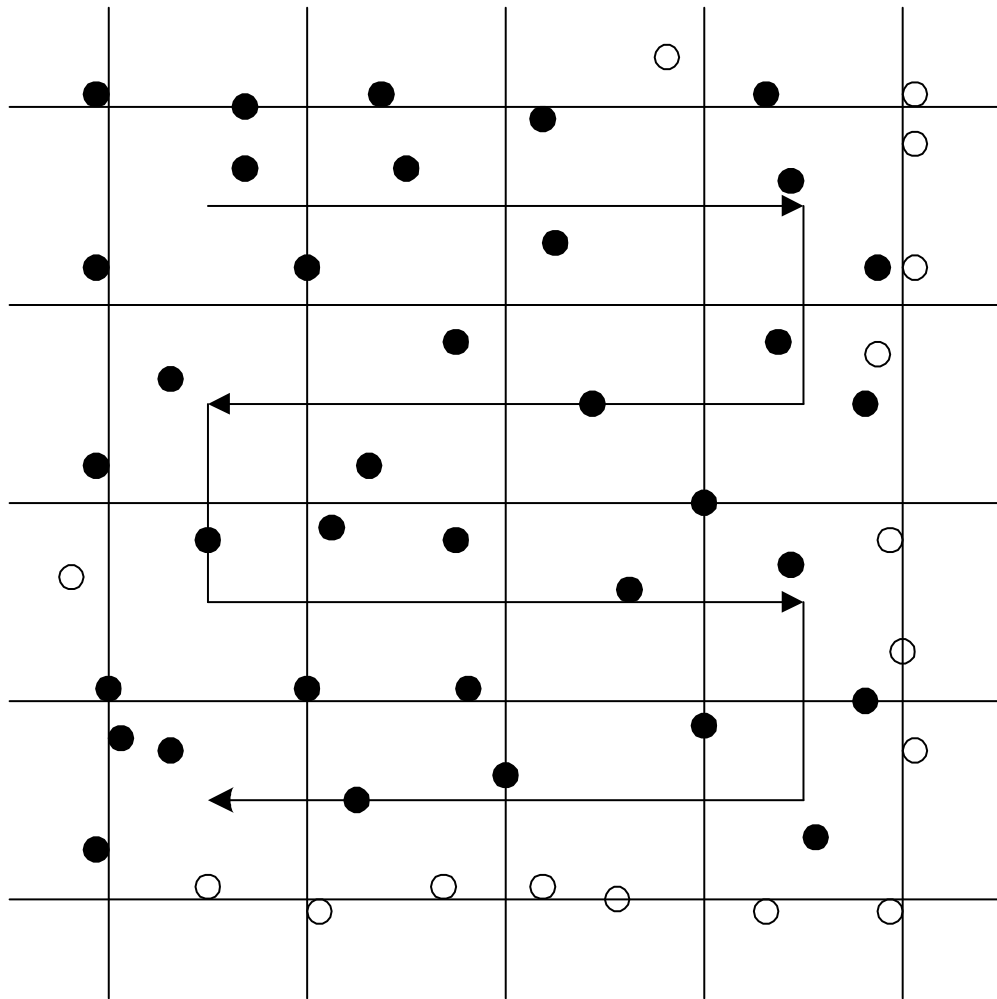


Figure 2. Manner of Counting Oocysts and Cysts in 1 Square mm. Dark organisms are counted and light organisms are omitted (after Miale, 1967).

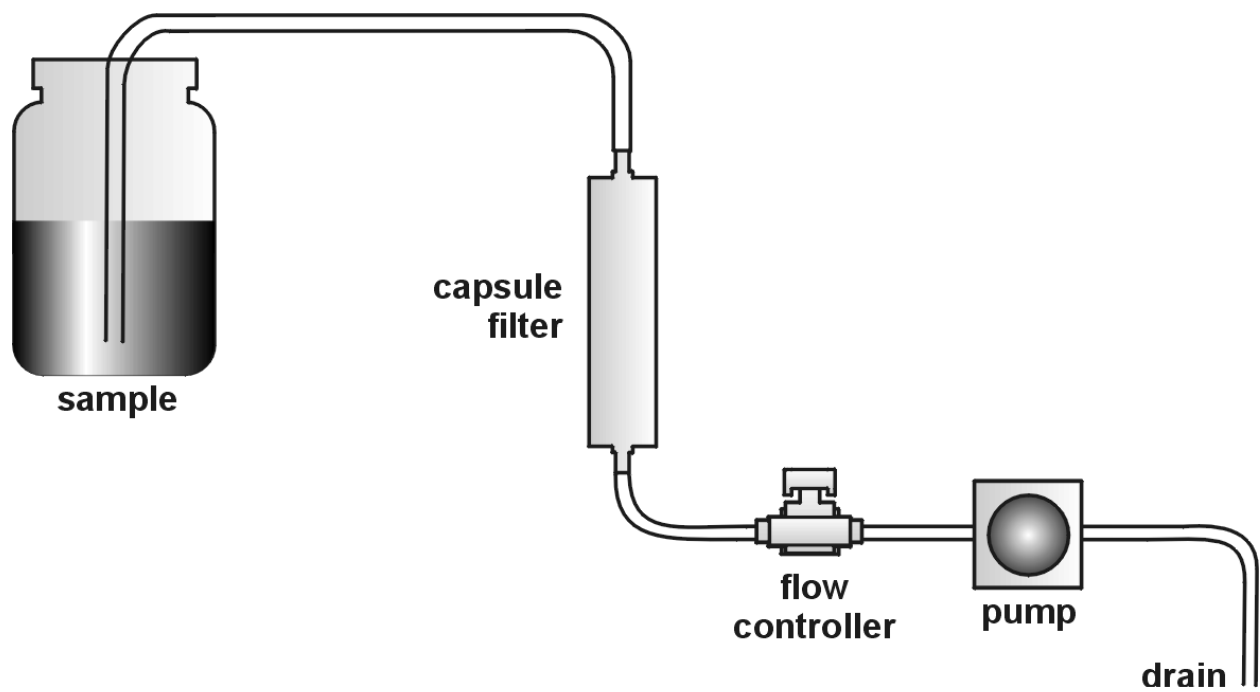


Figure 3. Laboratory Filtration System

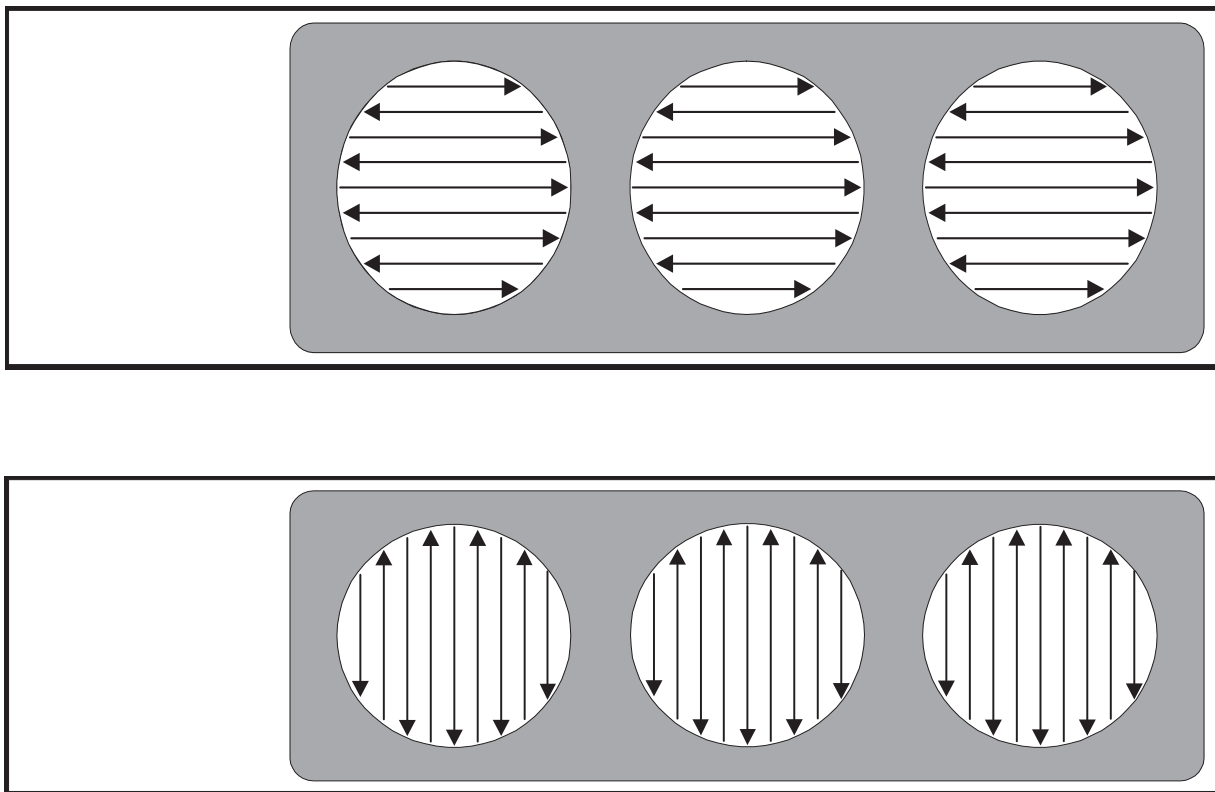


Figure 4. Methods for Scanning a Well Slide

22.0 Glossary of Definitions and Purposes

These definitions and purposes are specific to this method but have been conformed to common usage as much as possible.

22.1 Units of weight and measure and their abbreviations

22.1.1 Symbols

°C	degrees Celsius
μL	microliter
<	less than
>	greater than
%	percent

22.1.2 Alphabetical characters

cm	centimeter
g	gram
G	acceleration due to gravity
hr	hour
ID	inside diameter
in.	inch
L	liter
m	meter
MCS	microscope cleaning solution
mg	milligram
mL	milliliter
mm	millimeter
mM	millimolar
N	normal; gram molecular weight of solute divided by hydrogen equivalent of solute, per liter of solution
RSD	relative standard deviation
s _r	standard deviation of recovery
X	average percent recovery

22.2 Definitions, acronyms, and abbreviations (in alphabetical order)

~~Analyst—The analyst should have at least 2 years of college in microbiology or equivalent or closely related field. The analyst also should have a minimum of 6 months of continuous bench experience with *Cryptosporidium* and IFA microscopy. The analyst should have a minimum of 3 months experience using EPA Method 1622 and/or EPA Method 1623 and should have successfully analyzed a minimum of 50 samples using EPA Method 1622 and/or EPA Method 1623. The analyst must have at least 2 years of college lecture and laboratory course work in microbiology or a closely related field. The analyst also must have at least 6 months of continuous bench experience with environmental protozoa detection techniques and IFA microscopy, and must have successfully analyzed at least 50 water and/or wastewater samples for *Cryptosporidium* and *Giardia*. Six months of additional experience in the above areas may be substituted for two years of college.~~

Analyte—A protozoan parasite tested for by this method. The analytes in this method are *Cryptosporidium* and *Giardia*.

Axoneme—An internal flagellar structure that occurs in some protozoa, such as *Giardia*, *Spironucleus*, and *Trichomonas*.

Cyst—A phase or a form of an organism produced either in response to environmental conditions or as a normal part of the life cycle of the organism. It is characterized by a thick and environmentally resistant cell wall.

Flow cytometer—A particle-sorting instrument capable of counting protozoa.

Immunomagnetic separation (IMS)—A purification procedure that uses microscopic, magnetically responsive particles coated with an antibodies targeted to react with a specific pathogen in a fluid stream. Pathogens are selectively removed from other debris using a magnetic field.

Initial precision and recovery (IPR)—Four aliquots of spiking suspension analyzed to establish the ability to generate acceptable precision and accuracy. An IPR is performed prior to the first time this method is used and any time the method or instrumentation is modified.

Laboratory blank—See Method blank

Laboratory control sample (LCS)—See Ongoing precision and recovery (OPR) standard

Matrix spike (MS)—A sample prepared by adding a known quantity of organisms to a specified amount of sample matrix for which an independent estimate of target analyte concentration is available. A matrix spike is used to determine the effect of the matrix on a method's recovery efficiency.

May—This action, activity, or procedural step is neither required nor prohibited.

May not—This action, activity, or procedural step is prohibited.

Median bodies—Prominent, dark-staining, paired organelles consisting of microtubules and found in the posterior half of *Giardia*. In *G. intestinalis* (from humans), these structures often have a claw-hammer shape, while in *G. muris* (from mice), the median bodies are round.

Method blank—An aliquot of reagent water that is treated exactly as a sample, including exposure to all glassware, equipment, solvents, and procedures that are used with samples. The method blank is used to determine if analytes or interferences are present in the laboratory environment, the reagents, or the apparatus.

Must—This action, activity, or procedural step is required.

Negative control—See Method blank

Nucleus—A membrane-bound organelle containing genetic material. Nuclei are a prominent internal structure seen both in *Cryptosporidium* oocysts and *Giardia* cysts. In *Cryptosporidium* oocysts, there is one nucleus per sporozoite. One to four nuclei can be seen in *Giardia* cysts.

Oocyst—The encysted zygote of some sporozoa; e.g., *Cryptosporidium*. The oocyst is a phase or form of the organism produced as a normal part of the life cycle of the organism. It is characterized by a thick and environmentally resistant outer wall.

Ongoing precision and recovery (OPR) standard—A method blank spiked with known quantities of analytes. The OPR is analyzed exactly like a sample. Its purpose is to assure that the results produced by the laboratory remain within the limits specified in this method for precision and recovery.

Oocyst and cyst spiking suspension—See Spiking suspension

Oocyst and cyst stock suspension—See Stock suspension

Positive control—See Ongoing precision and recovery standard

Principle analyst—The principle analyst (may not be applicable to all monitoring programs) should have a BS/BA in microbiology or closely related field and a minimum of 1 year of continuous bench experience with *Cryptosporidium* and IFA microscopy. The principle analyst also should have a minimum of 6 months experience using EPA Method 1622 and/or EPA Method 1623 and should have analyzed a minimum of 100 samples using EPA Method 1622 and/or EPA Method 1623.

PTFE—Polytetrafluoroethylene

Quantitative transfer—The process of transferring a solution from one container to another using a pipette in which as much solution as possible is transferred, followed by rinsing of the walls of the source container with a small volume of rinsing solution (e.g., reagent water, buffer, etc.), followed by transfer of the rinsing solution, followed by a second rinse and transfer.

Reagent water—Water demonstrated to be free from the analytes of interest and potentially interfering substances at the method detection limit for the analyte.

Reagent water blank—see Method blank

Relative standard deviation (RSD)—The standard deviation divided by the mean times 100.

RSD—See Relative standard deviation

Should—This action, activity, or procedural step is suggested but not required.

Spiking suspension—Diluted stock suspension containing the organism(s) of interest at a concentration appropriate for spiking samples.

Sporozoite—A motile, infective stage of certain protozoans; e.g., *Cryptosporidium*. There are four sporozoites in each *Cryptosporidium* oocyst, and they are generally banana-shaped.

Stock suspension—A concentrated suspension containing the organism(s) of interest that is obtained from a source that will attest to the host source, purity, authenticity, and viability of the organism(s).